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PRINCIPAL INVESTIGATOR: Chawnshang Chang, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, NY 14627

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Chawnshang Chang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Rochester
Rochester, NY 14627

E-Mail: chang@URMC.rochester.edu

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13. ABSTRACT (Maximum 200 Words)

Data suggest androgen/androgen receptor (AR) may be involved in prostate cancer proliferation, but opposite roles of cell growth inhibition and apoptosis are documented. The detailed mechanism of how androgen/AR functions in apoptosis, remains unclear. Serine/threonine kinase (Akt) plays a role in promoting cell survival through anti-apoptotic effects. Akt was found active in prostate cancer LNCaP cells and plays an essential role for survival. Our preliminary data demonstrated Akt phosphorylates AR at Ser210 and Ser790. Mutation at Ser210 results in reversion of Akt-mediated suppression of AR transactivation. Activation of phosphatidylinositol-3-OH kinase/Akt pathway results in the suppression of AR target genes. Our hypothesis is that Akt may control androgen/AR-induced apoptosis by phosphorylating and inhibiting AR. Our Akt studies led into study of PTEN pathway. We have shown 1) via PI3K/Akt-dependent pathway, PTEN regulates AR activity in high passage LNCaP cells and suppresses AR activity in the early passage LNCP cells, 2) PI3K/Akt pathway promoted AR ubiquitylation, leading to AR degradation, and 3) restoration of AR function or PI3K/Akt pathway rescues cells from PTEN-induced apoptosis.

Even though we were unable to complete any of the Aim 4 our understanding of cross-talk between Akt and androgen/AR pathway in prostate cancer progression has been enhanced.

14. SUBJECT TERMS
androgen receptor, coregulator, gene expression/regulation, prostate cancer, cancer cell growth, pathology, Akt, PTEN pathway

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Chawnshang Chang
University of Rochester Medical Center
601 Elmwood Avenue, Box 626
Rochester, New York 14642

Tele: 585-275-9994
Fax: 585-756-4133
Chang@URMC.rochester.edu

DAMD17-02-1-0133 – Final report

TITLE: Suppression of androgen receptor transactivation by Akt kinase

INTRODUCTION:
Most data suggest androgen/AR may be involved in proliferation of prostate cancer, however opposite roles of androgen/AR in inhibition of cell growth and apoptosis are also documented. The detailed mechanism of how androgen/AR functions in apoptosis, however, remains unclear. A serine/threonine kinase (Akt) was demonstrated to play a role in promoting cell survival with anti-apoptotic effects. Akt was also found to be constitutively active in prostate cancer LNCaP cells and play an essential role for LNCaP survival. Our hypothesis is that Akt may control androgen/AR-induced apoptosis by phosphorylating and inhibiting AR. Our aims are 1) to prove that Akt can promote AR degradation via phosphorylation of AR *in vivo*, 2) to dissect the molecular mechanism by which Akt promotes AR protein degradation, 3) to determine whether Akt can suppress androgen/AR-induced cell growth inhibition and apoptosis, and 4) to generate site-specific phospho-AR antibodies and use these Abs to monitor the AR phosphorylation status and their relationship to the progression of prostate cancer in archival human tissues. Our project's success may enhance our understanding of cross-talk between Akt and androgen/AR pathway on prostate cancer progression.

BODY:
Our progress is summarized in the parts of the manuscripts published in the *J. Biol. Chem.* "Suppression vs induction of androgen receptor functions by the phosphatidylinositol 3-Kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers", by Hui-Kuan Lin, Yueh-Chiang Hu, Lin Yang, Saleh Altuwaijri, Yen-Ta Chen, Hong-Yo Kang and Chawnshang Chang. *J. Biol. Chem.* 19, 50902-50907, 2003, and in *Molecular Endocrinology* Regulation of Androgen Receptor Signaling by PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome 10) Tumor Suppressor through Distinct Mechanisms in Prostate Cancer Cells by Hui-Kuan Lin, Yueh-Chiang Hu, Dong Kun Lee, and Chawnshang Chang, *Mol. Endo.* 18, 2409-2423, 2004. The shortened abstracts are below, and the entire manuscripts are attached as Appendix A and B. There are no other publications related to this grant at present, however some of the data obtained may be presented in publications along with data from other projects in the PI's laboratory.

J. Biol. Chem Abstract: The phosphatidylinositol 3-kinase (PI3K)/Akt pathway controls several important biological functions, such as cell growth regulation, apoptosis, and migration. However, how PI3K/Akt controls androgen receptor (AR)-mediated prostate cancer cell growth remains unclear and controversial. Here, we demonstrate that the PI3K/Akt pathway regulates AR activity in a cell passage number-dependent manner, can suppress AR activity in androgen-dependent LNCaP cells with low passage numbers and also enhance AR activity in LNCaP cells with high passage numbers. We also demonstrate that insulin-like growth factor-1 (IGF-1)

can activate the PI3K/Akt pathway that results in the phosphorylation of AR at S210 and S790 to change the stability of AR protein. Together, our results demonstrate that the PI3K/Akt pathway may have distinct mechanisms to modulate AR functions in various stages of prostate cancer cells and a combined therapy of antiandrogens and anti-PI3K/Akt inhibitors may be worth consideration as future therapeutic approaches to battle the prostate cancer.

Narrative on Specific progress in the Aims.

Aim 1: To prove that Akt can promote AR degradation via phosphorylation of AR *in vivo*. Our preliminary studies indicated that Akt phosphorylates AR *in vitro* and could suppress AR transactivation in prostate cancer cells. Because phospho-inositol 3 kinase (PI3K). is an upstream activator of Akt, we applied the PI3K inhibitor LY294002 in LNCaP cells to block the PI3K/Akt pathway, to see whether AR expression and activity can be really influenced by this signaling pathway. Our studies in year 1 indicated that the blockade of this PI3K/Akt pathway causes increased AR expression and activity, proving the *in vivo* phosphorylation of AR by Akt through the PI3K activation pathway. See attached *J. Biol. Chem* manuscript. Our additional studies in 2003 led us into studies involving the PTEN pathway and its relationship to our Akt studies. See attached *Mol. Endo.* manuscript.

Aim 2: To dissect the molecular mechanism by which Akt promotes AR protein degradation. The same *in vivo* studies described in Aim 1 above are also mechanistic studies. See studies in *J. Biol. Chem.* manuscript. Here is a somewhat abbreviated abstract for submission to *Mol. Endo.* followed by some results/conclusions. Also in Results/conclusions find specific Figure references (the Figure numbers in bold shown refer to the Figures in the manuscript).

Abstract: Here we show that PTEN suppresses androgen receptor (AR) activity via a PI3K/Akt-independent pathway in the early passage number of prostate cancer LNCP cells. As androgen/AR plays important roles in prostate cancer progression, understanding the factors involved in the regulation of androgen/AR action may provide molecular targets for prostate cancer treatment. Here we demonstrate that PTEN regulates AR activity in low-passage number LNCaP cells via a PI3K/Akt-independent pathway and interacts directly with AR to suppress androgen-induced AR nuclear translocation. The interaction between AR and PTEN may expose the active site of the AR for the recognition of caspase-3, leading to AR degradation. In contrast, PTEN regulates AR activity in high passage number LNCaP cells via a PI3K/Akt-dependent pathway.

Results/Conclusions: (See Appendix B for all figures in manuscript) We also studied AR protein stability by pulse-chase labeling. As shown in Fig. 1A, **(Figure 5 C, Mol. Endo)** PTEN clearly reduced the half-life of newly synthesized [^{35}S]-AR 4- to 5-fold and accelerated AR degradation. Interestingly, when we replaced PTEN with either the dominant negative form of Akt (dAkt) or PI3K inhibitor LY294002, the results (Fig. 1B) **(Fig 1 E in Mol. Endo.)** indicated that dAkt and LY294002 did not promote AR degradation, ruling out the possibility that PTEN promotes AR degradation via regulation of the PI3/Akt pathway. These data strongly suggest that direct PTEN-AR protein-protein interaction may play major roles for the PTEN-promoted AR degradation. In contrast, in high passage number LNCaP cells (passage 65) where the PI3/Akt pathway becomes dominant (Fig. 2A and B) **(Figure 1C, D, and E, Mol. Endo),** PTEN-induced AR degradation was reversed by cAkt (Fig. 1B **(Figure 5E, Mol. Endo)** and 2B **(Figure 1E, Mol. Endo),**), suggesting that the suppressive effect of PTEN on AR involves Akt pathway and Akt might not promote AR ubiquitylation and degradation in high passage LNCaP cells.

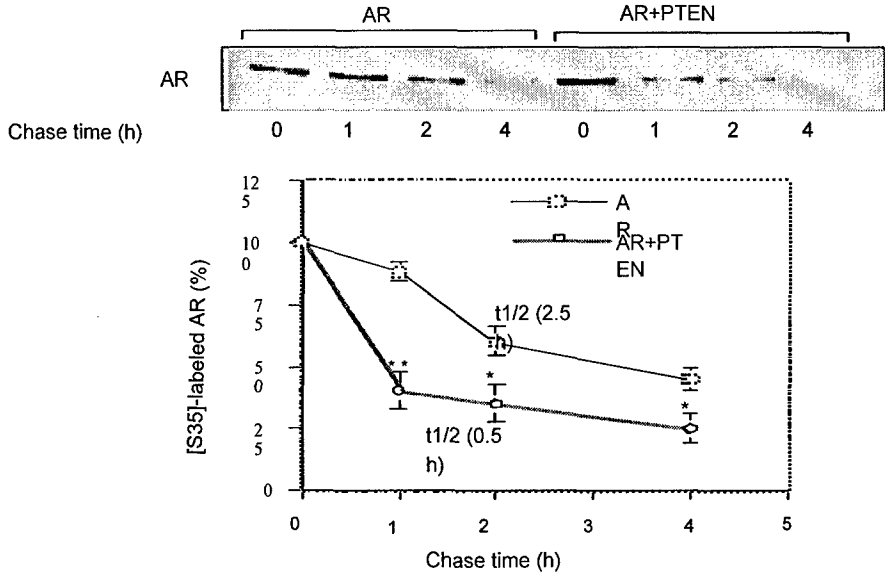
We reported recently that the PI3K/Akt pathway promoted AR ubiquitylation, leading to AR degradation by the 26 S proteasome (See attached *J. Biol. Chem.* manuscript). These data clearly suggest that both PTEN and the PI3K/Akt pathway can promote AR degradation via distinct mechanisms. How can PTEN negatively regulate the PI3K/Akt pathway and at same

time promote AR degradation? Since PI3K/Akt signaling promotes AR degradation, PTEN inhibition of this pathway would be expected to result in increased AR protein levels. It is possible that PTEN can go through both pathways by inhibition of PI3K/Akt-mediated AR degradation by the 26 S proteasome and caspase-3-mediated AR degradation. Yet the overall balance may favor the caspase-3-mediated AR degradation.

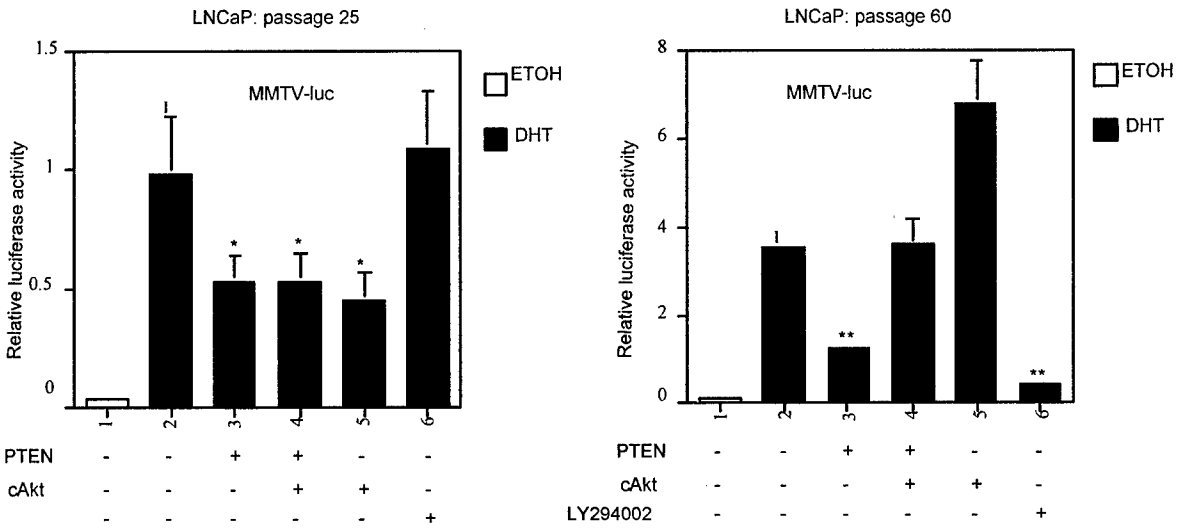
Aim 3: To determine whether Akt can suppress androgen/AR-induced cell growth inhibition and apoptosis.

(See Mol. Endo manuscript) The loss of PTEN expression in prostate LNCaP cells leads to constitutive activation of Akt (1). Akt is an important survival factor in a variety of cell types including LNCaP cells. Several lines of evidence have indicated that PI3K/Akt is able to suppress cell apoptosis induced by growth factor deprivation (2, 3, 4). Abrogation of PI3K/Akt activity by PI3K inhibitors causes LNCaP cell apoptosis (5, 6). On the other hand, the androgen/AR signal is thought to play important roles in the prostate cancer cell growth and survival, and this signal can protect cells from apoptosis in response to treatment of PI3K inhibitors (5, 7). Thus, the PI3K/Akt and the androgen/AR signaling pathways represent two major survival pathways in the LNCaP prostate cancer cells. As PTEN could repress the androgen/AR signal and PI3K/Akt pathway in LNCaP cells, we propose that inhibition of these two pathways by PTEN might contribute to PTEN-induced cell apoptosis in the LNCaP prostate cancer cells. This assertion was further supported by the observation that restoration of AR function or the PI3K/Akt pathway rescues cells from PTEN-induced apoptosis (Fig. 3).

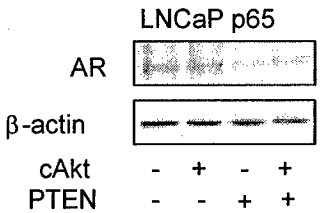
Fig. 1A, **(Figure 5 C, Mol. Endo)**



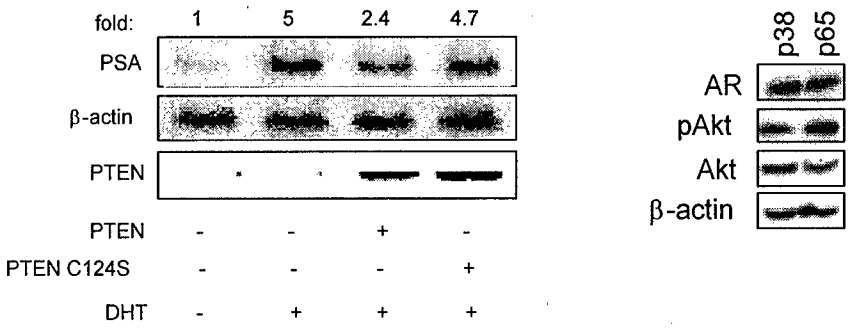
(Fig. 1B) **(Fig 1 E in Mol. Endo.)**



(Fig. 1C **(Figure 5E, Mol. Endo)**



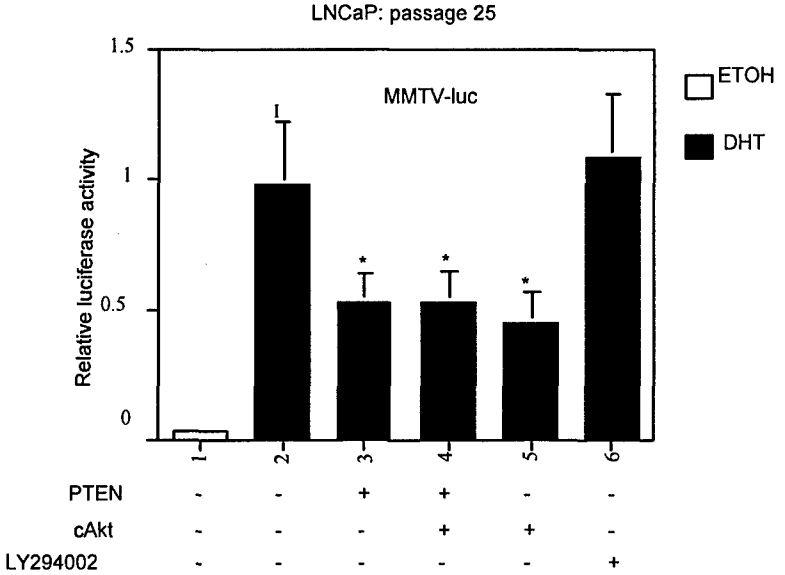
(Fig. 2A and B) **(Figure 1C, D, and E, Mol. Endo),**



Aim 3: To determine whether Akt can suppress androgen/AR-induced cell growth inhibition and apoptosis.

Fig. 3.

Androgen/AR induces apoptosis in prostate cancer PC-3 (AR)2 and PC-3(AR)6 cells.



Akt can suppress androgen-induced apoptosis in this cell model by inhibiting androgen receptor through directly phosphorylating androgen receptor.

The rest of the studies were done in prostate cancer LNCaP cells, in which loss of PTEN expression leads to constitutive activation of Akt (1). Akt is an important survival factor in a variety of cell types. PI3K and Akt is able to suppress cell apoptosis induced by growth factor deprivation (2, 3, 4). Abrogation of PI3K/Akt activity by PI3K inhibitor causes LNCaP cell apoptosis (5, 6). On the other hand, the androgen/AR signal is though to play important roles in the prostate cancer cell growth and survival, and this signal can protect cells from apoptosis in response to treatment of PI3K inhibitors (5, 7). In our submitted paper, (*J. Biol. Chem*, Induction of AR expression by FOXO3a, and their roles in apoptosis of LNCaP cells) , we found PI3K inhibitor did not lead to cell death but cell arrest under normal growth media culture conditions. Knockdown of AR by transfection of AR siRNA causes 51% of the cells to enter sub-G1 phase. Transfection of AR siRNA plus PI3K inhibitor further induces cell apoptosis; 65.7% cells underwent apoptosis. (Fig. 4D). Thus, the PI3K/Akt and the androgen/AR signaling pathways represent two major survival pathways in LNCaP prostate cancer cells.

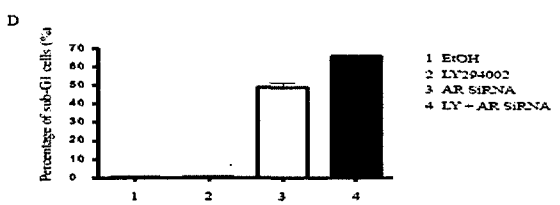


Figure 4: Flow cytometric analysis of LNCaP cells. (B) Effects of AR and LY294002 on cell cycle and apoptosis induction in LNCaP cells. LNCaP cells were transfected with AR siRNA or empty vector. After 24 h, cells were treated with 20 μM LY294002 for 24 h. The cells then were stained with propidium iodide to detect cell cycle and apoptosis as described in Materials and Methods. (C). Histograms of the cell cycle analysis. (D) Histograms of the sub-G1 population.
JBC, in submission

As PTEN could repress the androgen/AR signal and PI3K/Akt pathway in LNCaP cells, we propose that inhibition of these two pathways by PTEN might contribute to PTEN-induced cell apoptosis in the LNCaP prostate cancer cells. This assertion was further supported by the observation that restoration of AR function or the PI3K/Akt pathway rescues cells from PTEN-induced apoptosis (Fig. 5F see Molecular Endo 2004)

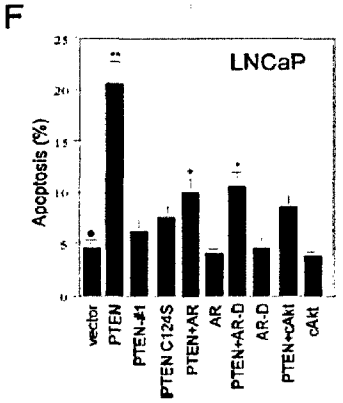


Fig. 5. Interaction between PTEN and AR Contributes to PTEN-Mediated Apoptosis and Suppression of AR Functions F, The LNCaP cells were transfected with plasmids, as indicated, for 16 h, and the medium was changed to 0.1% CDS media for 2 d. The cell apoptosis was determined by TUNEL assay. PTEN, but not PTEN-no. 1 (aa 1~107) or mutant PTEN-C124S, induced LNCaP cell apoptosis. Increased AR expression by transfection of AR, interrupting PTEN-AR interaction by AR-D, and overexpressing cAkt could rescue LNCaP cell apoptosis caused by PTEN. Data for luciferase activity and apoptosis are means ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.001$ *vs.* control (indicated as ●), Student's two-tailed *t* test.

Molecular Endo 2004, 18:2409-2423

Aim 4: To generate site-specific phospho-AR antibodies and use these Abs to monitor the AR phosphorylation status and their relationship to the progression of prostate cancer.

Several site-specific phospho-AR antibodies were tested for their specificity and to determine appropriate and efficient testing concentrations and procedures. However these prepared antibodies proved to be ineffective and/or inefficient. Last year we were hoping to have additional antibodies available for testing and studies on archival human tissue samples could proceed after the evaluations of the antibodies are completed. Unfortunately after several trials and experiments we found most of these antibodies were also ineffective and/or inefficient.. The only one that works is an anti-phospho-AR (ser^{210}) antibody. The anti-phospho-AR (ser^{210}) antibody (clone 156C135.2) was generated from the phospho-AR peptides (SGRAREADGAPTSSKD) according to the manufacturer's procedures (Androscience, San Diego, CA). As shown in Fig. 2B and 2C (See *J. Biol. Chem.* manuscript), The anti-phospho-AR (ser^{210}) antibody was used in the western blot analysis to support our hypothesis that activation of the PI3K/Akt pathway induces AR phosphorylation *in vivo*.

KEY RESEARCH ACCOMPLISHMENTS:

- Via a PI3K/Akt-dependent pathway PTEN regulates AR activity in high passage number LNCaP cells.
- PI3K/Akt pathway promoted AR ubiquitylation, leading to AR degradation by the 26 S proteasome.
- Via a PI3K/Akt-independent pathway PTEN suppresses androgen receptor (AR) activity in the early passage number of prostate cancer LNCP cells.
- Restoration of AR function or the PI3K/Akt pathway rescues cells from PTEN-induced apoptosis

• Site-specific antibodies evaluated with poor results, however others are in process of being developed, which also proved to be unsuccessful antibodies, with one exception as noted above in Aim 4.

REPORTABLE OUTCOMES:

• Two manuscripts associated with the proposal were (parts of the Figures and data were supported by this grant) published. (Note: again we apologize for neglecting to acknowledge this DOD grant.) "Suppression vs induction of androgen receptor functions by the phosphatidylinositol 3-Kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers." by Hui-Kuan Lin, Yueh-Chiang Hu, Lin Yang, Saleh Altuwaijri, Yen-Ta Chen, Hong-Yo Kang and Chawnshang Chang. 2003, *J. Biol. Chem.* 278, 50902-50907, and Regulation of Androgen Receptor Signaling by PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome 10) Tumor Suppressor through Distinct Mechanisms in Prostate Cancer Cells by Hui-Kuan Lin, Yueh-Chiang Hu, Dong Kun Lee, and Chawnshang Chang, 2004, *Mol. Endo.* 18, 2409-2423.

• One extra J. Biol. Chem. paper with one Figure supported by this grant is under revision and will include the Grant Number in the acknowledgements.

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Dr. Chawnshang Chang
Dr. Hiroshi Miyamoto
Dr. Hui-Kuan Lin (received his Ph.D. in 2003)
Dr. Huei-Ju Ting (received her Ph.D. in 2004)
Dr. Tong-Zu Liu (Postdoctoral Fellow-2004)
Jiann-Jai Lai (Graduate student-2004)

CONCLUSIONS:

As a summary, we ask how to interpret these findings and what is the physiological role of increased AR function after PI3K/Akt is blocked? We found that removal of androgens in LNCaP cells resulted in increased levels of active phosphorylated Akt. Thus, we believe that the AR and PI3K/Akt signaling both appear to be important proliferation and survival factors in prostate cancer cells, and seem to antagonize each other to maintain the cell homeostasis. The AR activity can be induced by LY294002 to play a dominant proliferation role to compensate for the loss of PI3K/Akt signaling. Our additional studies in 2003 led us into studies involving the PTEN pathway and its relationship to our Akt studies. Although we had expected to have tissue studies with antibodies by now, we find we are unable to complete and barely started these studies due our failure to find suitable effective antibodies with the one exception as noted above in Aim 4.

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Manuscript A: "Suppression vs induction of androgen receptor functions by the phosphatidylinositol 3-Kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers", by Hui-Kuan Lin, Yueh-Chiang Hu, Lin Yang, Saleh Altuwaijri, Yen-Ta Chen, Hong-Yo Kang and Chawnshang Chang. 2003, *J. Biol. Chem.* 278, 50902-50907

Manuscript B: "Regulation of Androgen Receptor Signaling by PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome 10) Tumor Suppressor through Distinct Mechanisms in Prostate Cancer Cells. by Hui-Kuan Lin, Yueh-Chiang Hu, Dong Kun Lee, and Chawnshang Chang, 2004, *Mol. Endo.* 18, 2409-2423.

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Suppression *Versus* Induction of Androgen Receptor Functions by the Phosphatidylinositol 3-Kinase/Akt Pathway in Prostate Cancer LNCaP Cells with Different Passage Numbers*

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Hui-Kuan Lin‡, Yueh-Chiang Hu‡, Lin Yang‡, Saleh Altuwaijri‡, Yen-Ta Chen‡§, Hong-Yo Kang‡§, and Chawnshang Chang‡¶

From the ‡George Whipple Laboratory for Cancer Research, Departments of Pathology, Urology, and Radiation Oncology and The Cancer Center, University of Rochester Medical Center, Rochester, New York 14642 and §Center for Menopause and Reproductive Medicine Research, Chang Gung University, Kaohsiung 833, Taiwan

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway controls several important biological functions, such as cell growth regulation, apoptosis, and migration. However, the way in which PI3K/Akt controls androgen receptor (AR)-mediated prostate cancer cell growth remains unclear and controversial. Here, we demonstrate that the PI3K/Akt pathway regulates AR activity in a cell passage number-dependent manner. Specifically, PI3K/Akt pathway can suppress AR activity in androgen-dependent LNCaP cells with low passage numbers. In contrast, it can also enhance AR activity in LNCaP cells with high passage numbers. Furthermore, we also demonstrate that insulin-like growth factor-1 can activate the PI3K/Akt pathway that results in the phosphorylation of AR at Ser^{210} and Ser^{790}. The consequence of these events may then change the stability of AR protein. Together, our results demonstrate that the PI3K/Akt pathway may have distinct mechanisms to modulate AR functions in various stages of prostate cancer cells and that a combined therapy of antiandrogens and anti-PI3K/Akt inhibitors may be worth considering as a future therapeutic approach to battle prostate cancer.

Prostate cancer is the second leading cause of cancer-related death among men in the United States. The normal prostate and prostate cancers at early stages require androgen for growth and survival. In addition to androgen signaling, which plays an essential role in survival of prostate cancer, the phosphatidylinositol 3-kinase (PI3K)[1]/Akt pathway represents another important survival signal for prostate cancer cells. It appears that these two pathways can compensate for each other in growth regulation of prostate cancer LNCaP cells, because androgen treatment can rescue cells from apoptosis induced by application of PI3K inhibitors (1). Furthermore, activation of the PI3K/Akt pathway protects cells from apoptosis induced by serum starvation and androgen deprivation (2).

Recent rapid progress of the PI3K/Akt signal pathway studies, as well as its influence on the androgen receptor (AR)-mediated prostate cancer growth, has resulted in many exciting yet controversial results. Here we address these controversial results by summarizing Akt-AR-related results and provide new data, as well as possible explanations for the distinct roles of the PI3K/Akt pathway in AR-mediated prostate cancer growth. Particular emphases will be: 1) Akt suppresses *versus* induces AR activity, 2) Akt phosphorylation sites on AR protein, and 3) promotion of AR degradation by the PI3K/Akt pathway.

EXPERIMENTAL PROCEDURES

Reagents—pCDNA3 cAkt (3) and mutant AR S210A/S790A were described previously (4). pCDNA3-PTEN was a gift from Dr. Charles L. Sawyers, and pGEX-KG-PTEN was from Dr. Frank B. Furnari. Insulin-like growth factor-1 (IGF-1) and LY294002 was from Calbiochem. 5α-Dihydrotestosterone (DHT), doxycycline (Dox), and cycloheximide were from Sigma. The anti-AR polyclonal antibody, NH27, was produced as described previously (3). The mouse monoclonal PTEN and prostate-specific antigen (PSA) antibodies and the goat polyclonal β-actin antibody were from Santa Cruz Biotechnology. The mouse monoclonal Akt and phospho-Akt (Ser^{473}) antibodies were purchased from Cell Signaling.

Cell Culture and Transfections—DU145, PC-3, and COS-1 cell lines were maintained in Dulbecco's minimum essential medium containing penicillin (25 units/ml), streptomycin (25 μg/ml), and 10% fetal calf serum (FCS). LNCaP cells were maintained in RPMI 1640 with 10% FCS. Transfections were performed using SuperFect™ according to standard procedures (Qiagen).

Luciferase Reporter Assays—Luciferase reporter assay was as described previously with some modifications (5). The cells were transfected with plasmids in 10% charcoal-stripped serum (CSS) medium for 16 h and then treated with ethanol or 10 nM DHT for 16 h. The cells were lysed, and luciferase activity was detected by the dual luciferase assay according to standard procedures (Promega). Mouse mammary tumor virus-luciferase (MMTV-luc), which contains the AR response elements, was used as an AR transactivation reporter. The results were normalized by *Renilla* luciferase activity (pRL-SV40-luc), and the data represent means ± S.D. from triplicate sets of three independent experiments.

LNCaP Stable Transfectants—For the Dox-inducible system, PTEN was released from pGEX-KG-PTEN using EcoRI digestion and inserted into pBIG2i vector. The LNCaP cells were transfected with pPIB2i PTEN for 24 h. The cells were selected using 100 μg/ml hygromycin. Individual colonies were picked and grown until 70% confluent followed by 4 μg/ml Dox treatment. The positive clones were confirmed by Western blot analysis.

Generation of an Anti-phospho-AR Antibody (Ser^{210})—The phospho-AR peptide (SGRAREADGAPTSSKD) was generated and used for generation of anti-phospho-AR (Ser^{210}) antibody (clone 156C135.2) according to the manufacture's procedures (AndroScience, San Diego, CA).

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¶ To whom correspondence should be addressed. E-mail: chang@urmc.rochester.edu; Website: www.urmc.rochester.edu/ChangARlab.

[1] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; AR, androgen receptor; cAkt, constitutively active form of Akt; CSS, charcoal-stripped serum; DHT, 5α-dihydrotestosterone; Dox, doxycycline; E3 ligase, ubiquitin-protein isopeptide ligase; FCS, fetal calf serum; IGF, insulin-like growth factor; MMTV-luc, mouse mammary tumor virus-luciferase; PSA, prostate-specific antigen; PTEN, phosphatase and tensin homolog deleted on chromosome ten.

Immunoprecipitation and Western Blot Analysis—Immunoprecipitation and Western blotting were performed as previously described (3). Cell extracts (1 mg) were immunoprecipitated with the indicated antibody. The immunocomplexes were subjected to 8% SDS-PAGE and immunoblotted with the indicated antibody.

Cell Growth Assay—LNCaP cells (2×10^4) with different passage numbers were grown in 12-well plates, transfected with parent vector or the constitutively active form of Akt (cAkt), and cultured in 10% CSS medium after 3 h of transfection. Cells were stained by trypan blue on different days, as indicated, and cell numbers were determined by direct counting on hemacytometers. The data represent means ± S.D. from triplicate sets of three independent experiments.

RESULTS AND DISCUSSION

Cell-specific and Passage-dependent Effect of PI3K/Akt Signaling on AR Activity—The PI3K/Akt pathway plays an important role in cell growth, survival, adhesion, and migration in a variety of cell types. In prostate cancer LNCaP cells, the PI3K/Akt pathway is a dominant survival signal pathway for cells, and inhibition of this pathway by PI3K inhibitors leads to cell growth arrest and apoptosis (6). Recently, it has been demonstrated that the PI3K/Akt pathway regulates AR activity and phosphorylation (3, 7). Although activation of the PI3K/Akt pathway suppresses AR activity in androgen-independent prostate cancer DU145 cells (3), other reports also demonstrated that the PI3K/Akt pathway enhances AR activity in androgen-dependent prostate cancer LNCaP cells (7, 8). Although the detailed mechanisms of these differential effects remain unclear, it is possible that different cell types may have differential PI3K/Akt effects on AR activity, which led to our examination of various prostate cancer cells.

Interestingly, we found that the PI3K/Akt pathway could regulate AR activity in a passage-dependent manner in LNCaP cells. cAkt suppressed AR activity in low passage number LNCaP cells (passage number 25) (Fig. 1*A*, *P25*) but enhanced AR activity in high passage number LNCaP cells (Fig. 1*B*, *P60*), in reporter gene assays. It should be noted that the reporter gene activation by androgen was much higher in higher passage LNCaP cells (Fig. 1, compare *panel B* with *A*). The reason for this phenomenon is currently unknown. This may suggest that some factors that preferentially exist or are over-expressed in higher passage LNCaP cells may contribute to the enhancement of this androgen response. Blockage of the PI3K/Akt pathway by LY294002 slightly enhanced AR activity in low passage number LNCaP cells but suppressed AR activity in high passage number LNCaP cells (Fig. 1, *A* and *B*, *4th lanes on right*). Although LY294002 has been widely used as a PI3K inhibitor, we cannot rule out the possibility that at 20 μM this reagent may affect other kinases that influence AR activity. We performed a Western blot assay to examine the role of the PI3K/Akt pathway in regulating AR target gene expression. Even though LY294002 only marginally enhanced AR activity in low passage LNCaP cells in the reporter gene assays (Fig. 1*A*), it apparently increased androgen-induced PSA expression, an AR target gene, in low passage number LNCaP cells (Fig. 1*C*). Similar to the reporter gene assay, LY294002 suppressed PSA expression in high passage number LNCaP cells (Fig. 1*C*). Moreover, cAkt reduced androgen-induced PSA expression in low passage number LNCaP cells but slightly enhanced PSA expression in high passage number LNCaP cells (Fig. 1*D*). These results suggest that distinct passage numbers of LNCaP cells might influence the effects of the PI3K/Akt effect on AR activity. Using PC-3 cells, Thompson *et al.* (9) also demonstrated that the PI3K/Akt pathway could suppress AR activity, which is consistent with our data (Fig. 1*A*) and with early reports using DU145 cells as the cell model (3). Together, these results demonstrate that the effects of the PI3K/Akt signaling pathway on AR activity may change with different prostate cancer cell lines and within the same cell line at different passage numbers.

At early stages, prostate cancer cells may need androgen signaling for growth and survival. Androgen ablation or antiandrogen treatment may lead to cell growth arrest and apoptosis of these androgen-sensitive cancer cells (1). The basal activity of the PI3K/Akt pathway in the early stage prostate tumors is lower and may not be adequate to play a major role in the maintenance of prostate cancer cell growth and survival in the absence of concurrent androgen signaling. However, androgens may become less important factors for tumor cell growth and survival in late stage prostate cancer. In contrast, tumor cells at this later stage have higher basal activity of the PI3K/Akt pathway, which may contribute to the development of prostate cancer progression by preventing cells from apoptosis (10).

To support the above hypothesis, we found that the low passage LNCaP cells possess a low basal level of Akt activity (Fig. 1*E*). In contrast, high passage LNCaP cells show a strong basal Akt activity (Fig. 1*E*). Our data show that Akt negatively modulates AR activity in low passage LNCaP cells (Fig. 1*A*), suggesting that LNCaP cells at this early stage require more androgen to compensate for the suppressive effect of the low basal Akt activity and that the low basal Akt activity may not be sufficient to provide the survival signal necessary for maintenance of cell growth and survival.

To determine whether Akt is a determining factor for the androgen reliance of LNCaP cell growth, we cultured LNCaP cells in CSS medium lacking androgen to compare the growth pattern of LNCaP cells at different passage numbers in the presence or absence of cAkt. As expected, early passage LNCaP cells, with low basal activity of Akt, showed little cell growth in the CSS medium (Fig. 1*F*), suggesting that the androgens are important for cell growth. In contrast, high passage LNCaP cells, with higher basal Akt activity, grew much faster than early passage LNCaP cells (Fig. 1*F*), suggesting less dependence on the androgens. Elevation of the basal Akt activity by transfection of cAkt significantly increased the LNCaP cell growth at both cell passages, although the effect of cAkt was more profound in the early passage LNCaP cells (Fig. 1*F*). Thus, the Akt signal may be a key factor in driving LNCaP cell growth and survival at this late stage with weaker androgen reliance.

Considering the biphasic effect of PI3K/Akt and androgen signaling on the progression of prostate cancer, we found that androgen ablation therapy, which removes most of the androgens available for prostate tumors, may result in increased activation of the PI3K/Akt pathway, promoting tumor cell growth and survival. This hypothesis is further supported by a recent report (11) showing that the PI3K/Akt pathway is elevated in LNCaP cells cultured in androgen-depleted medium. It is possible that increased PI3K/Akt signaling upon loss of androgen signaling may contribute to the failure of androgen ablation therapy at later stages of prostate cancer. For this reason, using a combination therapy that includes androgen ablation at early stages and suppression of the PI3K/Akt pathway at later stages may provide a better strategy for battling prostate cancer.

The Effect of PI3K/Akt Signaling on AR Phosphorylation—AR is a phosphoprotein, and its activity can be modulated by phosphorylation (12). We demonstrated that activation of PI3K/Akt pathways by IGF-1 in COS-1 cells induces AR phosphorylation *in vivo* (3). The *in vitro* kinase assay further revealed that Akt, but not PI3K, phosphorylates AR at Ser^{210} and Ser^{790} residues, which are the Akt consensus phosphorylation sites (3). Overexpression of cAkt, but not the kinase-dead Akt

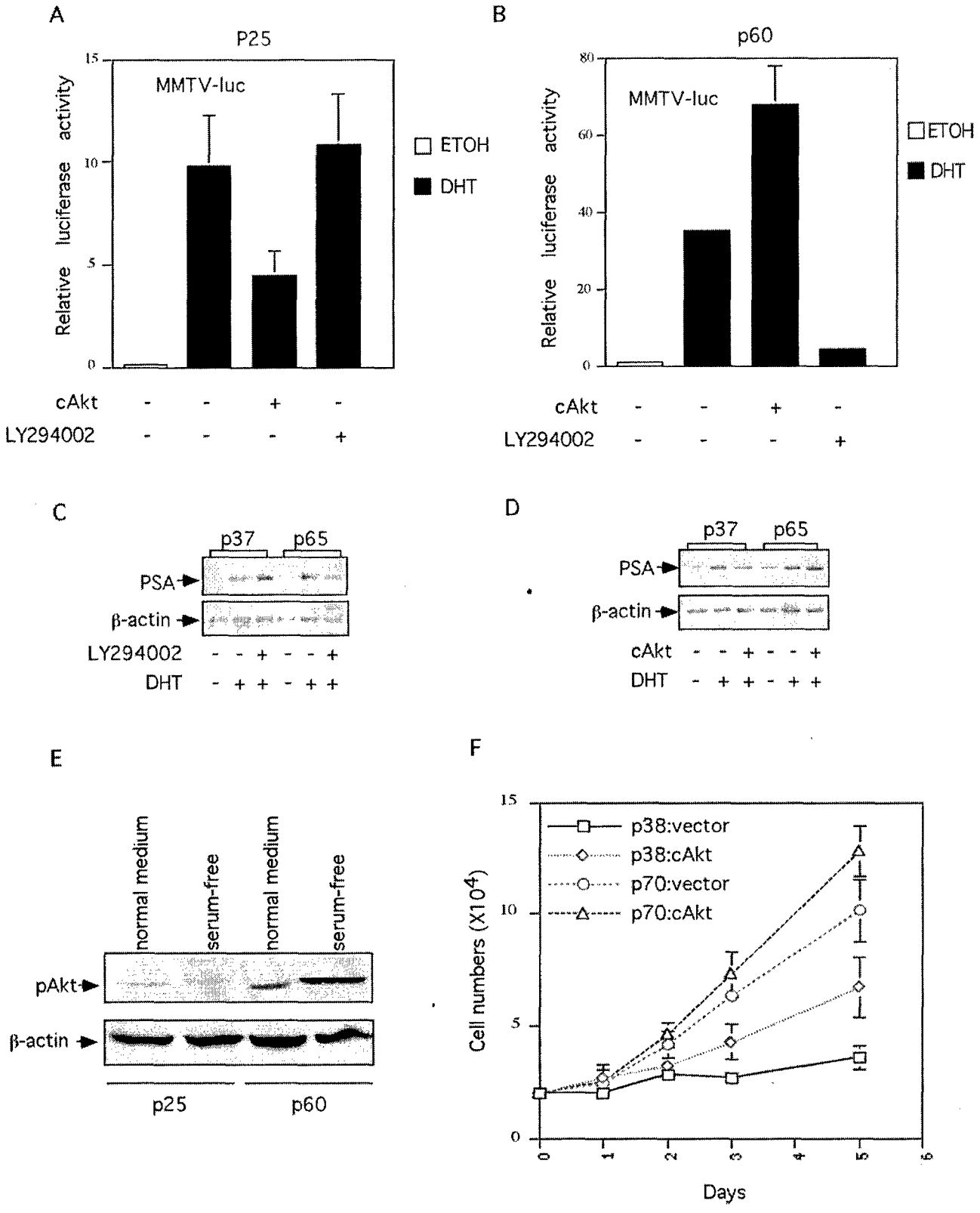

FIG. 1. **Passage-dependent effect of the PI3K/Akt pathway on AR transactivation in LNCaP cells.** *A*, LNCaP cells (passage 25 (*P25*)) were transfected with MMTV-luc along with plasmids, as indicated, for 16 h, and cells were then treated with EtOH or 10 nM DHT in the presence or absence of 20 μM LY294002 for 24 h. The cells were harvested for luciferase assay. *B*, the same experiment as described in *A* was carried out with LNCaP cells at passage 60 (*P60*). *C*, LNCaP cells at different passage numbers were cultured in 10% CSS for 24 h, treated with 20 μM LY294002 10 min prior to 10 nM DHT treatment for another 24 h, and harvested for Western blot assay. *D*, LNCaP cells at different passage numbers were transfected with vector or cAkt for 24 h, and cells were treated with EtOH or 10 nM DHT for another 24 h, followed by harvesting cells for Western blot assay. *E*, different passage numbers of LNCaP cells were cultured in the 10% FCS medium or serum-free medium for 2 days, and the cells were harvested for Western blot analysis. Akt activity is determined by the levels of Akt phosphorylation (*pAkt*) using anti-phospho-Akt (Ser^{473}) antibody. *F*, LNCaP cells at different passages were transfected with vector or cAkt and cultured in CSS medium. Cells were stained by trypan blue on different days, and cell numbers were determined as described under "Experimental Procedures."

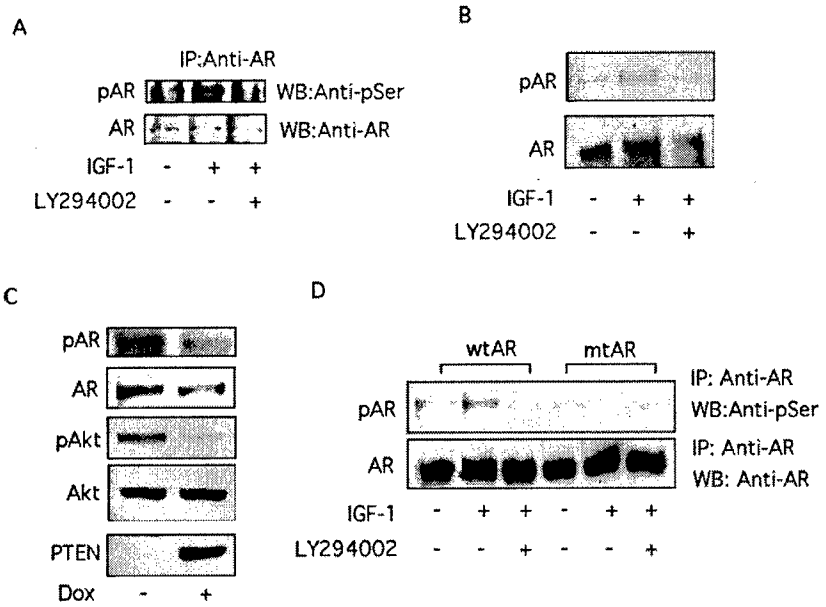

FIG. 2. **Activation of the PI3K/Akt pathway induces AR phosphorylation *in vivo*.** *A*, LNCaP cells at passage 38 were serum-starved for 2 days, incubated with 20 μM LY294002 for 30 min prior to treatment with 100 μg/ml IGF-1 for 4 h, and then harvested for immunoprecipitation (*IP*) with AR antibody. *WB*, Western blot; *Anti-pSer*, anti-phosphoserine antibody. *B*, LNCaP cells at passage 38 were treated as described in *A* and harvested for Western blot analysis. Total AR protein was blotted using an anti-AR antibody (*AR*), and AR phosphorylation was detected using an anti-phospho-AR (Ser^{210}) antibody (*pAR*). *C*, PTEN-inducible LNCaP cells at passage 40 were cultured in 10% FCS, treated with 4 μg/ml Dox for 24 h, treated with 100 μg/ml IGF-1 for 4 h, and then harvested for Western blot analysis. *D*, COS-1 cells were transfected with wild-type (*wtAR*) or mutant AR (*mtAR*, $Ser^{210}Ala/Ser^{790}Ala$) for 16 h, serum-starved for 24 h, and then incubated with 20 μM LY294002 for 30 min prior to treatment with 100 μg/ml IGF-1 for 4 h. The cells were then harvested for immunoprecipitation with anti-AR antibody and Western blot analysis. *Anti-pSer*, anti-phosphoserine antibody.

mutant (dAkt), induced AR phosphorylation *in vivo*, and mutations at the consensus serine residues reduced Akt-mediated AR phosphorylation (3). Consistent with our results, Wen *et al.* (7) also found that Akt associated with AR and phosphorylated AR at Ser^{210} and Ser^{790} *in vitro*.

We and others (3, 7) and have found that Akt can phosphorylate AR at Ser^{210} and Ser^{790}. However, Gioeli *et al.* (13) found that Akt fails to phosphorylate AR at Ser^{210} and Ser^{790} in LNCaP cells. They also found that PI3K inhibitor LY294002 did not change the levels of AR phosphorylation in a two-dimensional gel electrophoresis assay (13). These contrasting results may be because of the use of different cell lines (COS-1 *versus* LNCaP cells) to test AR phosphorylation under various transfection and treatment conditions. Alternatively, another explanation for the discrepancy may be that the overexpression of Akt via transient transfection may produce protein levels that are far higher than that seen under physiological conditions.

To determine whether gene overexpression was a confounding factor in the interpretation of our AR phosphorylation assays, we used IGF-1 to activate endogenous PI3K/Akt and therefore mimic physiological conditions. As shown in Fig. 2*A*, we demonstrated that IGF-1 treatment induced AR phosphorylation in LNCaP cells (passage number 38), and adding the PI3K inhibitor LY294002 blocked IGF-1-mediated AR phosphorylation, suggesting that the PI3K/Akt pathway is involved in the phosphorylation of AR. Using a site-specific anti-phosphoserine AR antibody, AR phosphorylation at Ser^{210} was detected when LNCaP cells were treated with IGF-1 (Fig. 2*B*). Moreover, using the Dox-inducible system we generated the inducible PTEN clone, a tumor suppressor that antagonizes the PI3K/Akt pathway (14), in LNCaP cells at passage number 40. PTEN expression induced by Dox treatment inhibited Akt activation and AR phosphorylation at Ser^{210} (Fig. 2*C*). IGF-1 also induced wild-type AR phosphorylation in COS-1 cells (Fig. 2*D*, *wtAR*), and LY294002 blocked the IGF-1-mediated phosphorylation. In contrast, IGF-1 did not induce phosphorylation of the mutant AR (S210A/S790A), in which two Akt consensus sites were mutated from Ser to Ala (Fig. 2*D*, *mtAR*). These data therefore strongly support our early findings that the PI3K/Akt pathway activated by IGF-1 mediates AR phosphorylation at Ser^{210} and Ser^{790} (3). In contrast, Gioeli *et al.* (13) did not add growth factors such as IGF-1 to activate the PI3K/Akt pathway. It is therefore possible that the level of the Akt activity in LNCaP cells may not be sufficient to induce AR activity, given that the basal level of Akt activity is low in early passage LNCaP cells (Fig. 1*E*) in which AR phosphorylation by Akt may not occur and may require the addition of growth factors to amplify the PI3K/Akt signal.

Regulation of AR Protein Turnover by the PI3K/Akt Pathway—AR controls several biological functions, including prostate cell growth and apoptosis (12). However, the mechanism by which AR maintains its stability for proper function remains largely unknown. Growing evidence implies that AR may be degraded by the ubiquitin-proteasome pathway (15–17). In support of this notion, we have recently demonstrated that activation of the PI3K/Akt pathway induces AR ubiquitylation and subsequent degradation by the 26 S proteasome (4). The effect of Akt on AR ubiquitylation and degradation seems to be dependent on AR phosphorylation, because activation of Akt did not induce ubiquitylation or degradation of mutant AR, which lacks Akt-mediated phosphorylation. Interestingly, the AR mutant was remarkably stable compared with wild-type AR, suggesting that phosphorylation of AR by Akt reduces AR stability (4).

Mdm2, a Ring Finger protein, consists of an E3 ligase and suppresses p53 activity by regulation of ubiquitylation and degradation of p53 (18, 19). In addition to regulation of p53 function, Mdm2 can also regulate AR activity via regulation of ubiquitylation and degradation of the AR (4). We further identified Mdm2 as an E3 ligase for AR and a mediator for Akt-induced AR ubiquitylation and degradation (4). AR protein

normally undergoes degradation several hours after its synthesis in cells. However, the signals responsible for AR turnover remain unclear. Based on our data, we propose that the PI3K/Akt/Mdm2 pathway represents an important mechanism to control AR turnover rate. When LNCaP cells are cultured in normal medium, growth factors such as IGF-1 can activate the PI3K/Akt pathway, which may then be responsible for the turnover of AR protein. In support of this hypothesis, blockage of the PI3K/Akt pathway by LY294002 in LNCaP cells leads to increased AR protein levels (4).

Because the PI3K/Akt pathway differentially regulates AR activity in different passage numbers of LNCaP cells (Fig. 1, *A–D*), we next determined whether the PI3K/Akt pathway has a distinct effect on AR degradation in these cells. cAkt down-regulated AR protein levels in low passage LNCaP but slightly enhanced AR protein levels in high passage LNCaP cells (Fig. 3*A*). In contrast, LY294002 enhanced AR protein levels in low passage LNCaP cells but slightly reduced AR protein levels in high passage LNCaP cells (Fig. 3*B*). To prove the role of Akt in regulation of AR degradation directly, we examined the effect of Akt on AR protein stability. Overexpression of cAkt in low passage LNCaP cells led to accelerated AR degradation (Fig. 3*C, left panel*). cAkt did not promote AR degradation in high passage LNCaP cells but slightly enhanced AR stability (Fig. 3*C, right panel*), which indeed correlated with the effect of PI3K/Akt on AR transcriptional activity in Fig. 1, *A–D*, and AR protein levels in Fig. 3, *A* and *B*. These results suggest that the PI3K/Akt pathway induces AR degradation in low passage LNCaP cells but not in high passage LNCaP cells.

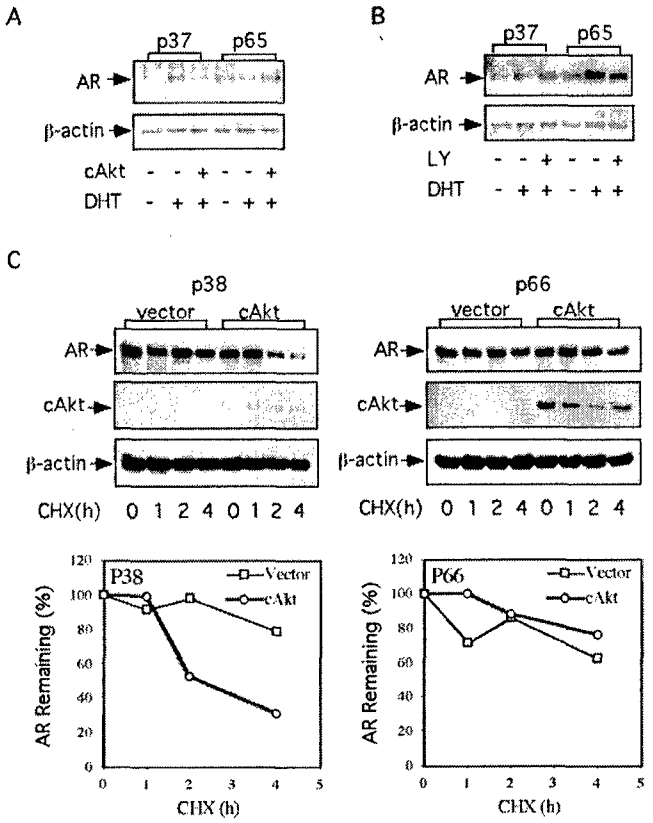

FIG. 3. **Distinct regulation of AR protein degradation by the PI3K/Akt pathway at various passage numbers of LNCaP cells.** *A*, LNCaP cells at different passage numbers were transfected with vector or cAkt for 24 h, and cells were treated with EtOH or 10 nM DHT for another 24 h followed by harvesting for Western blot assay. *B*, LNCaP cells at different passage numbers were cultured in 10% CSS medium for 24 h, treated with 20 μM LY294002 10 min prior to 10 nM DHT treatment for another 24 h, and harvested for Western blot assay. *C*, LNCaP cells at different passage numbers were transfected with vector or cAkt for 24 h, and cells were treated with 20 μg/ml cycloheximide (*CHX*) for different times, as indicated, in 10% FCS medium followed by harvesting for Western blot assay.

Exactly how the cell passage number affects PI3K/Akt modulation of AR activity remains unclear. However, it is possible that the variant basal Akt activity levels among cells of different passages may be a key factor contributing to this phenomenon. Alternatively, different cell contexts may exist in LNCaP cells of different passage numbers contributing to the modulating effect of the PI3K/Akt pathway on AR activity. Because Mdm2 is a downstream effector of the PI3K/Akt pathway, it would be useful to determine whether the levels of Mdm2 in various passage numbers of LNCaP cells are significantly different. A more global assay, such as proteomics, may be required to elucidate the factors that may contribute to this phenomenon.

Summary—On the basis of this study and our previous reports (3, 4) we propose a model for the PI3K/Akt pathway action on the regulation of AR activity in prostate cancer LNCaP cells (Fig. 4). The PI3K/Akt pathway exhibits a cell passage-dependent regulation of AR activity. In low passage LNCaP cells, the basal activity of PI3K/Akt signaling is low and cells are strongly dependent on androgen signaling for growth and survival. However, in high passage LNCaP cells, the basal activity of the PI3K/Akt pathway is high and cells are less dependent on androgen signaling. The PI3K/Akt pathway not only provides the growth and survival signals for prostate cancer cells, but it also enhances AR activity in high passage LNCaP cells via an unknown mechanism.

Several important questions have been raised throughout this study. First, what are the factors that determine the differential effects of the PI3K/Akt pathway on AR activity in different passage numbers of LNCaP cells? Second, what is the molecular mechanism by which the PI3K/Akt pathway enhances AR activity in the high passage LNCaP cells? Future studies should focus on these issues, and systematic analysis is required to solve these puzzles. Finally, the PI3K/Akt pathway

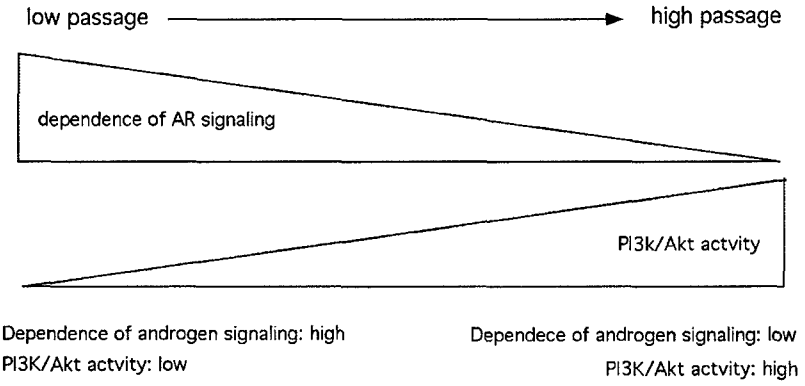

FIG. 4. **Model for PI3K/Akt pathway on AR signaling in prostate LNCaP cells.** In low passage LNCaP cells, the basal activity of PI3K/Akt signaling is low, and cells are strongly dependent on androgen signaling for growth and survival. In contrast, in high passage LNCaP cells, the basal activity of the PI3K/Akt pathway is high, and cells are less dependent on androgen signaling. The PI3K/Akt pathway not only provides the growth and survival signals for prostate cancer cells but also enhances AR activity in high passage LNCaP cells via an unknown mechanism.

provides a survival and growth signal for prostate cancer cells and induces AR activation in the presence or absence of androgen. Given its activation during prostate cancer progression, PI3K/Akt signaling may represent a new chemotherapeutic target with the potential to be particularly effective. A therapy that suppresses the PI3K/Akt pathway combined with classic androgen ablation therapy could reach the maximal effect in the battle against prostate cancer.

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Regulation of Androgen Receptor Signaling by PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome 10) Tumor Suppressor through Distinct Mechanisms in Prostate Cancer Cells

HUI-KUAN LIN, YUEH-CHIANG HU, DONG KUN LEE, AND CHAWNSHANG CHANG

George Whipple Laboratory for Cancer Research, Departments of Urology, Pathology, Radiation Oncology, and The Cancer Center, University of Rochester, Rochester, New York 14642

Defects in the *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene have been found in many human cancers including breast and prostate. Here we show that PTEN suppresses androgen receptor (AR) activity via a phosphatidylinositol-3-OH kinase/Akt-independent pathway in the early passage numbers prostate cancer LNCaP cells. We provide the direct links between PTEN and androgen/AR signaling by demonstrating that AR directly interacts with PTEN. The interaction between PTEN and AR inhibits the AR nuclear translocation and promotes the AR protein degradation that result in the suppression of AR transactivation and induction of apoptosis. The minimum interaction peptide within AR (amino acids 483–651) disrupts the interaction of PTEN with AR and reduces the PTEN effect on AR transactivation and apoptosis. Genetic approaches using PTEN-null mouse embryonic fibroblasts (MEFs) further demonstrate that both AR expression and AR activity were much higher in PTEN-null MEFs than wild-type MEFs, and reintroducing PTEN into PTEN-null MEFs dramatically reduced AR protein levels and AR activity. Interestingly, we also found that PTEN could suppress AR activity via the phosphatidylinositol-3-OH kinase/Akt-dependent pathway in the higher passage number LNCaP cells, because restoration of Akt activity blocks the effect of PTEN on AR activity. Together, these contrasting PTEN effects on AR activity in the same prostate cancer cell line with different passage numbers suggest that PTEN, via distinct mechanisms, differentially regulates AR in various stages of prostate cancers. (*Molecular Endocrinology* 18: 2409–2423, 2004)

THE ANDROGEN RECEPTOR (AR), a transcription factor, belongs to the nuclear receptor superfamily (1, 2). Once bound to androgen, AR translocates into the nucleus, leading to activation of its target genes (3). AR consists of the amino-terminal region that is involved in transcriptional activation, the DNA-binding domain (DBD), the hinge region that contains the nuclear localization signal, and the ligand-binding domain (LBD) that is involved in androgen binding and receptor dimerization (3). It is generally accepted that AR plays an important role in the development of the reproductive organs and in progression of prostate cancer (3–5). Maximal or proper androgen action may require the interaction of AR with several coregulators (6), such as ARA70 (7, 8), ARA55 (9, 10), ARA54 (11, 12), and steroid receptor coactivator-1 (13).

The tumor suppressor gene *PTEN* (phosphatase and tensin homolog deleted on chromosome 10), located at chromosome 10q23, is one of the most frequently mutated genes linked to a variety of human cancers (14–20). Germline mutations in *PTEN* cause the autosomal dominant inherited cancer syndromes such as Cowden's disease, which is associated with an elevated risk for malignant cancers (21). Loss of PTEN expression is frequently found in prostate cancer cell lines and tumor specimens (22). Mice with a heterozygous mutant *PTEN* develop prostate epithelial hyperplasia and dysplasia (23). Mice with inactivation of one allele of *PTEN* in combination with loss of the cyclin-dependent kinase (CDK)n1b (encoding $p27^{Kip1}$) gene have an acceleration of spontaneous neoplastic transformation and develop prostate carcinoma (24). Interestingly, mice deficient in CDKn1b do not develop prostate cancer (25–27), suggesting that PTEN and $p27^{Kip1}$ cooperate in prostate cancer suppression in the mouse. These results indicate that loss of PTEN

Abbreviations: aa, Amino acid; AR, androgen receptor; CDK, cyclin-dependent kinase; CDS, charcoal dextran-stripped serum; DBD, DNA-binding domain; DHT, 5α-dihydrotestosterone; Dox, doxycycline; ER, estrogen receptor; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GST, glutathione-*S*-transferase; LBD, ligand-binding domain; MEF, mouse embryonic fibroblast; MMTV-luc, mouse mammary tumor virus luciferase; NLS, nuclear localization signal; PI3K, phosphatidylinositol-3-OH-kinase; *PTEN*, phosphatase and tensin homolog deleted on chromosome 10; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate end-labeling; WT, wild-type.

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function may be a key event in prostate cancer progression.

Recent studies demonstrated that PTEN regulates not only cell growth and apoptosis, but also controls cell adhesion and migration (28–30). Whereas the PTEN sequence suggests that it may be a dual specificity phosphatase that includes lipid phosphatase and protein phosphatase activity, its protein substrates remain largely unknown. Recently, several groups have reported that the phosphatidylinositol-3-OH kinase (PI3K)/Akt pathway is negatively regulated by PTEN through its phospholipid 3-phosphatase activity (16, 17, 31–33). Whereas the PI3K/Akt-dependent pathway is the most popular model for PTEN action, other signaling pathways were also suggested (34). For instance, dPTEN regulates cell growth and proliferation in *Drosophila* through the PI3K/Akt-dependent and -independent pathway (34). Furthermore, using mouse embryonic fibroblasts (MEFs) from PTEN-null mice, Wu and coworkers (35). showed that PTEN can physically interact with p53 and regulate protein stability and transcriptional activity without its phosphatase activity, indicating that PTEN regulates p53 function independent of the PI3K/Akt pathway. As a consequence, loss of one allele of *PTEN* dramatically accelerates tumor formation of the p53 heterozygous mouse.

As androgen/AR plays important roles in prostate cancer progression, understanding the factors involved in the regulation of androgen/AR action may provide molecular targets for prostate cancer treatment. Here we demonstrate that PTEN regulates AR activity in low-passage number LNCaP cells via a PI3K/Akt-independent pathway and interacts directly with AR to suppress androgen-induced AR nuclear translocation. The interaction between AR and PTEN may expose the active site of the AR for the recognition of caspase-3, leading to AR degradation. In contrast, PTEN regulates AR activity in high passage number LNCaP cells via a PI3K/Akt-dependent pathway.

RESULTS

PTEN Suppresses AR Transactivation Involving the Pathways Other Than PI3K/Akt

The PTEN tumor suppressor induces cell apoptosis in a variety of cell types including prostate cancer cells. However, the molecular mechanism underlying PTEN-induced apoptosis in prostate cancer cells remains unclear. We were interested in testing the potential linkage between PTEN and androgen/AR signaling. To test this hypothesis, we determined the effect of the PTEN on AR transactivation using mouse mammary tumor virus-luciferase (MMTV-luc) as an AR reporter. PTEN suppressed AR transactivation in a dose-dependent manner in androgen-dependent prostate LNCaP cells and in androgen-independent prostate cancer PC-3 cells and DU145 cells (Fig. 1A). Interestingly, PTEN C124S, a PTEN mutant without phosphatase activity, still can suppress AR activity in DU145, PC-3, and LNCaP cells, but to a lesser extent (Fig. 1A). To rule out the possibility that the suppression of AR by PTEN might come from the suppressive effect of PTEN on general transcriptional machinery, we used pGL3-control vector (Promega Corp., Madison, WI) and pG5-Lucifase (a GAL4 reporter) as controls to demonstrate that PTEN has little or enhanced effect on these control luciferase vectors after being normalized with pRL-SV40 internal control used in all samples (Fig. 1B). Northern blot analysis further confirmed that PTEN could suppress androgen-induced expression of prostate-specific antigen, an endogenous AR target gene, in prostate cancer LNCaP cells (Fig. 1C).

It is generally believed that PTEN exerts its role in tumor suppression by negatively regulating the PI3K/Akt pathway. We next determined whether PTEN affects AR activity via the regulation of the PI3K/Akt pathway in different passage numbers of LNCaP cells. The protein expression levels of AR and Akt are comparable between the low and high passages of LNCaP cells, but the basal Akt activity in high passage number LNCaP was much higher than in low passage number LNCaP cells (Fig. 1D). Interestingly, in a low passage number of LNCaP cells (passage 25), PTEN suppresses AR activity via a PI3K/Akt-independent pathway, as addition of the constitutively active form of Akt (cAkt) does not reverse the suppressive effect of PTEN on AR activity (Fig. 1E, *left panel,* lane 4). Interestingly, cAkt, like PTEN, also suppressed AR activity in low passage number LNCaP cells (Fig. 1E, *left panel*, lane 5). PI3K/Akt inhibitor, LY294002, did not significantly enhance AR activity (Fig. 1E, *left panel*, lane 6) perhaps due to a low basal activity of Akt in such cells (Fig. 1D). However, restoration of Akt activity completely reversed the PTEN suppression of AR activity in high passage number LNCaP cells (Fig. 1E, *right panel*, lane 3), suggesting that PTEN can also regulate AR signaling via a PI3K/Akt-dependent pathway in LNCaP cells with different passage numbers. Unlike its suppression of AR activity in the lower passage number LNCaP cells, cAkt enhanced AR activity in the high passage number LNCaP cells (Fig. 1E. *right panel*, lane 5). Taken together, these contrasting results suggest that PTEN can regulate AR activity via the PI3K/Akt-dependent and -independent pathways in prostate cancer LNCaP cells at different growth stages.

PTEN Interacts with AR *in Vitro* and *in Vivo*

Because PTEN regulates AR activity via a PI3K/Akt-independent pathway in the early-passage LNCaP cells, we hypothesized that PTEN might function via direct interaction with AR. Indeed, our glutathione-*S*-transferase (GST) pull-down assay results indicated that PTEN could interact with AR in the presence or absence of androgen (Fig. 2A). Among several nuclear receptors we tested, we found that PTEN binds preferentially to AR and estrogen receptor (ER), as compared with glucocorticoid receptor (data not shown),

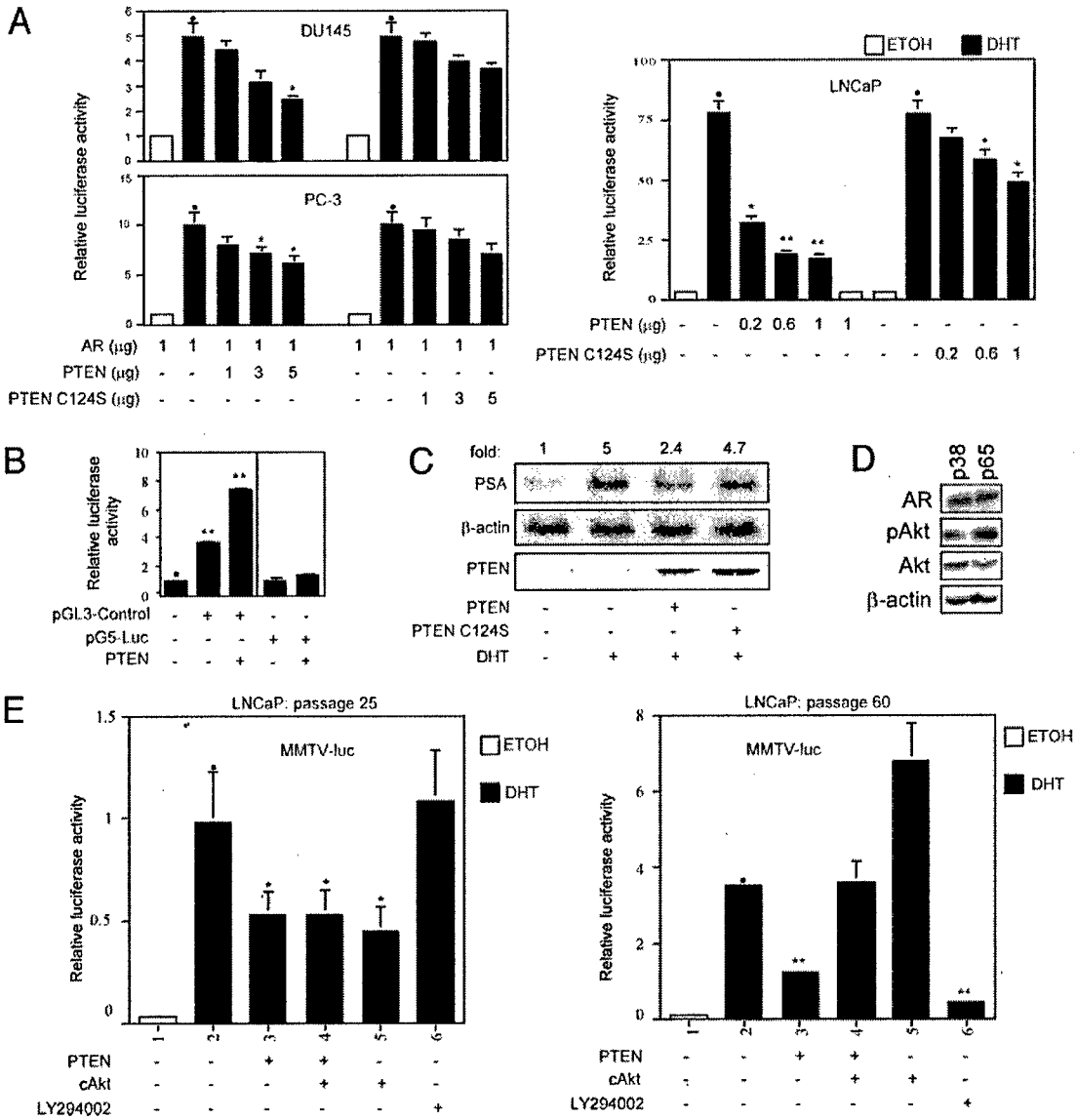

Fig. 1. PTEN Suppresses AR Transactivation Involving Pathways Other than PI3K/Akt

A, The LNCaP, PC-3, or DU145 cells were transfected with plasmids, as indicated, in 10% CDS media for 16 h and treated with ethanol (ETOH) or 10 nM DHT for another 16 h. The cells were harvested and assayed for luciferase activity using MMTV-luc as a reporter. B, LNCaP cells were transfected with plasmids, as indicated, in 10% FCS media for 32 h. pGL3-basic was used as control vector (lane 1). The cells were harvested and assayed for luciferase activity. C, LNCaP cells were transfected with plasmids, as indicated, in 10% CDS media for 24 h and then treated with DHT for 24 h. The cells were harvested for Northern blot analysis. D, Akt activity is higher in high-passage number of LNCAP cells. Different passages (passage 38 *vs.* 65) of LNCaP cells were cultured in 10% FCS media and harvested for Western blot analysis. E, LNCaP cells (passage 25) or LNCaP cells (passage 60) were transfected with MMTV-luc along with plasmids, as indicated, for 16 h, and cells were then treated with ETOH or 10 nM DHT in the presence or absence of LY294002 for 16 h. The results were normalized by pRL-SV40 activity and the data are represented as means ± SD of three independent experiments. [*, $P < 0.05$; **, $P < 0.001$ *vs.* control (indicated as ●), Student's two-tailed *t* test].

progesterone receptor (data not shown), or the retinoid X receptor (Fig. 2A).

To map the AR interaction domains on PTEN, the plasmids encoding a set of PTEN fragments fused with GST were constructed for the GST pull-down assays. The AR was able to interact with GST-PTEN-no. 2 [amino acids (aa) 107–252], where the phosphatase domain is located, but not with GST-PTEN-no. 1 (aa 1–107) or GST-PTEN-no. 3 (aa 253–403) (Fig. 2, B and C). Further peptide mapping revealed that PTEN-PTP (aa 110–163) containing the phosphatase domain is sufficient for interacting with AR (Fig. 2C).

Studies of the PTEN-interacting domain on AR indicated that the AR-DBD (aa 486–651) and AR-DBD plus LBD (AR-DBD-LBD) (aa 552–918), but not the AR amino-terminal region (AR-N) (aa 34–560) or AR-LBD

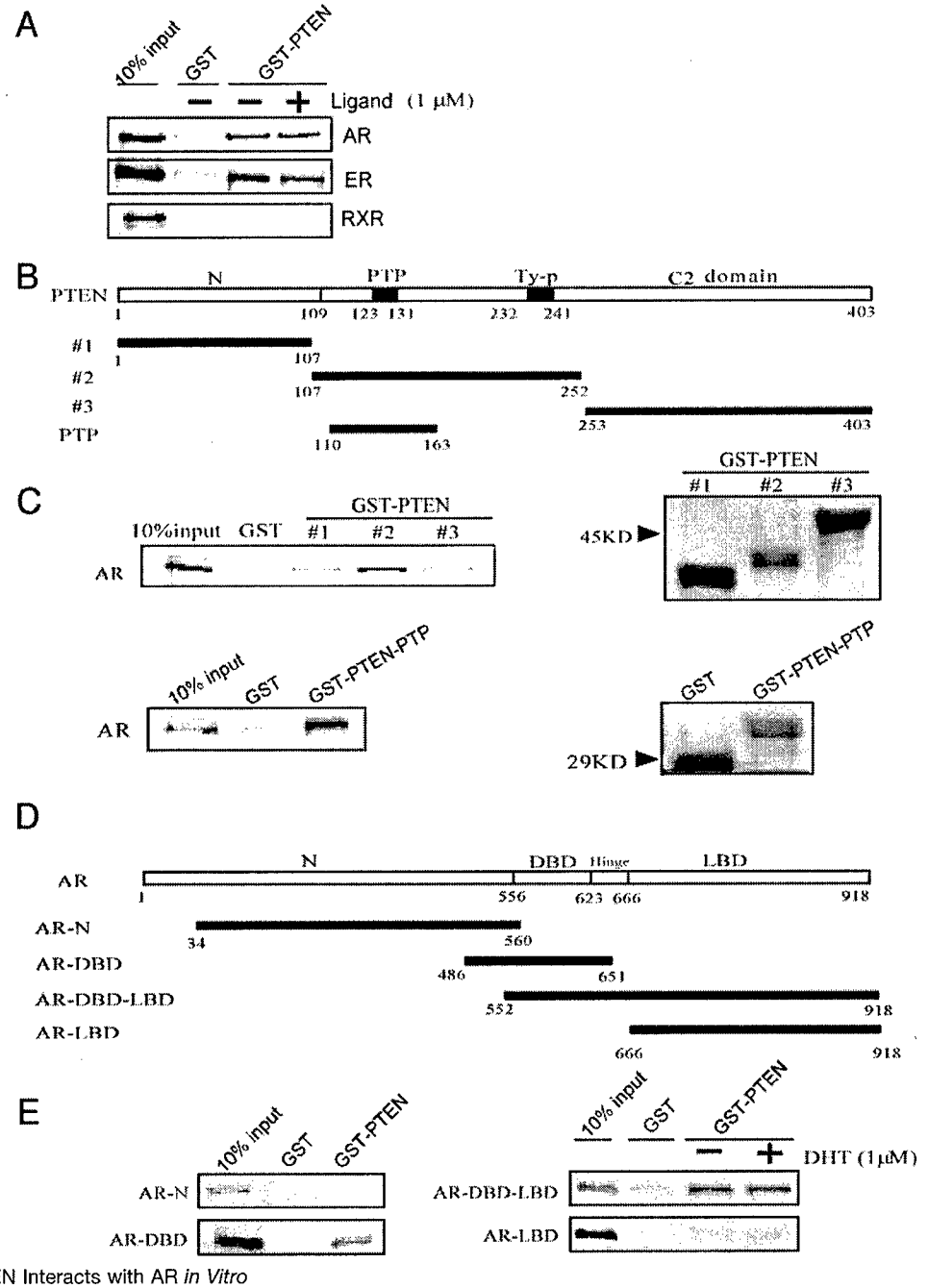

Fig. 2. PTEN Interacts with AR *in Vitro*

A, GST or GST-PTEN incubation with the ^{35}S-labeled AR, ER, or retinoid X receptor (RXR) for 2 h in the presence or absence of the ligand. The bound proteins were analyzed by SDS-PAGE, followed by autoradiography. B, Representative diagram of PTEN deletion mutants. PTP domain, protein tyrosine phosphatase domain; Ty-p, tyrosine phosphorylation domain. C, ^{35}S-labeled AR was incubated with different PTEN deletion mutants. The nearly equivalent aliquots of PTEN deletion mutants used are shown in the *right panel*. D, Representative diagram of AR deletion mutants. E, GST or GST-PTEN was incubated with different AR deletion mutants.

(aa 666–918), were able to interact with PTEN (Fig. 2, D and E). The GST pull-down assay results therefore suggest that AR can interact with PTEN (aa 110–163) via its DBD (aa 552–651).

To further confirm the physiological interaction between AR and PTEN by coimmunoprecipitation, we established PTEN-stable LNCaP cells, using the doxycycline (Dox)-inducible system. Dox treatment induced expression of PTEN or PTEN C124S in several clones (PTEN-C1, PTEN-C2, PTEN C124S-C4, and PTEN C124S-C8, Fig. 3A). AR could be coimmunoprecipitated with PTEN, when we used cell lysates from PTEN-C1 cells (Fig. 3B). To rule out the possibility that PTEN antibody may cross-react with AR, we dem-

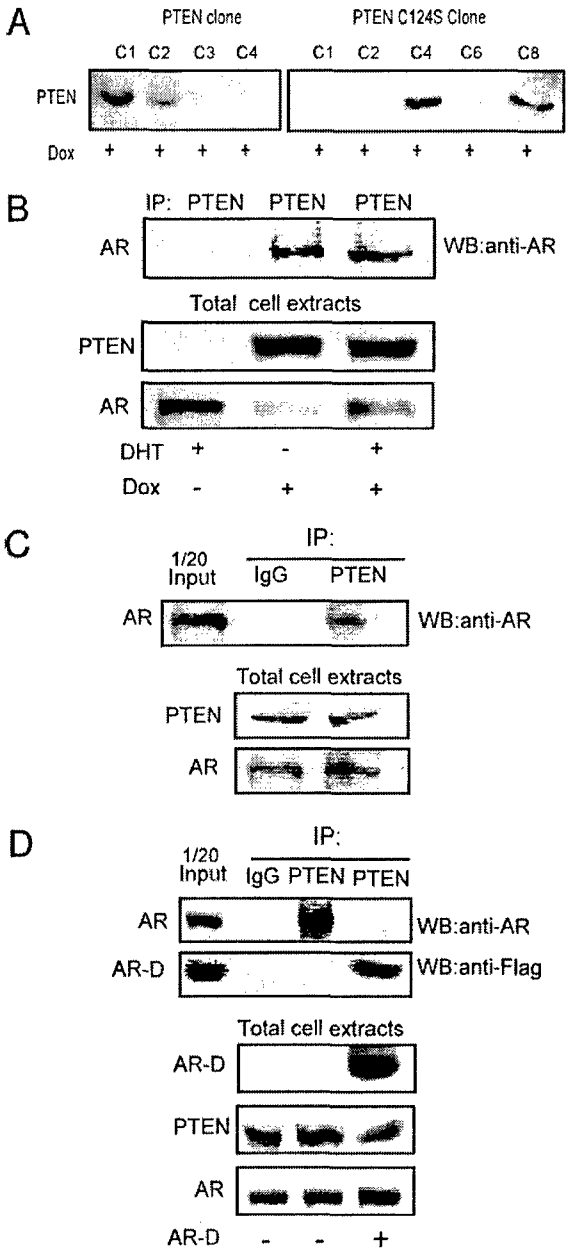

Fig. 3. PTEN Interacts with AR *in Vivo*

A, The establishment of stable PTEN and PTEN C124S clones in LNCaP cells using Dox-inducible system. The cells were treated with 4 μg/ml Dox for 24 h and harvested for Western blot analysis using PTEN antibody. PTEN and PTEN C124S expression can be induced with Dox treatment in Clone C1 and C2, and C4 and C8, respectively. B, AR exists in the PTEN immunocomplex in LNCaP cells overexpressing PTEN. The stable PTEN clone (PTEN-C1) was treated with or without 4 μg/ml Dox in 10% CDS media for 24 h and treated with ethanol or 10 nM DHT for another 24 h. The cells were harvested for immunoprecipitation (IP) assay with normal mouse PTEN antibody, followed by Western blotting with AR antibody. The total cell lysates were subjected to Western blotting with PTEN and AR antibodies. C, Endogenous association between PTEN and AR in CWR22R cells. The IP and Western blot methods used are the same as described in panel B except that the cell lysates were from the CWR22R cells. D, The PTEN-AR interaction is inhibited by AR-D in CWR22R cells. The cells cultured in RPMI medium with 10% FCS were transfected with vector or FLAG-tagged AR-D for 48 h. Cells were then harvested for immunoprecipitation (IP) assay with normal mouse IgG or PTEN antibody, followed by Western blotting with AR or FLAG antibodies. The total cell lysates were subjected to Western blotting with FLAG, PTEN, and AR antibodies.

onstrated that PTEN antibody did not pull down AR, using parental LNCaP cells, which express AR but not PTEN (data not shown). To further prove that endogenous PTEN can interact with the endogenous AR in the prostate cancer cell line, we applied the CWR22R cell line (36, 37), which endogenously expresses both AR and PTEN (Fig. 3C), for coimmunoprecipitation with PTEN antibody. The results showed that AR could be detected in the PTEN-immunoprecipitated complex (Fig. 3C). To further determine whether the DBD plus the hinge region of AR (aa 486–651, AR-D) can interact with PTEN, we transfected AR-D into CWR22R cells for coimmunoprecipitation with PTEN antibody. We found AR-D could be found in PTEN-immunoprecipitated complex (Fig. 3D). Interestingly, AR-D could also prevent endogenous PTEN from binding to AR in CWR22R cells (Fig. 3D). These results suggest that AR can physiologically interact with PTEN through the AR-D region in prostate cancer cells.

PTEN Colocalizes with AR and Inhibits AR Nuclear Translocation

The interaction between PTEN and AR was also analyzed by the subcellular colocalization study, using fluorescence immunostaining. As shown in Fig. 4A, the fluorescent FITC-stained PTEN was located mainly in the cytosol, but small amounts of PTEN were also found in the nucleus. Similar to the FITC-stained PTEN, Texas Red-stained AR was also located mainly in the cytosol in the absence of androgen, but androgen treatment caused AR nuclear translocation (Fig. 4A). Figure 4B further demonstrates that PTEN could colocalize with AR in the presence or absence of androgen. Interestingly, we found that PTEN significantly blocked AR nuclear translocation in response to androgen and increased the AR retention (from 4% to 38%) in the cytosol. In contrast, PTEN C124S showed only a slight inhibition of AR nuclear translocation (Fig. 4C). Similar results were also obtained in LNCaP cells with stable transfection of PTEN (passage 40). As shown in Fig. 4D, Dox-induced PTEN expression in the PTEN-stable PTEN-C2 cells could inhibit the AR nuclear translocation. In contrast, Dox showed little effect on the AR nuclear translocation in the parental pBIG2i cells. These results suggest that PTEN may be able to bind to AR and prevent the translocation of cytosolic AR into the nucleus, which may then result in suppression of AR transactivation.

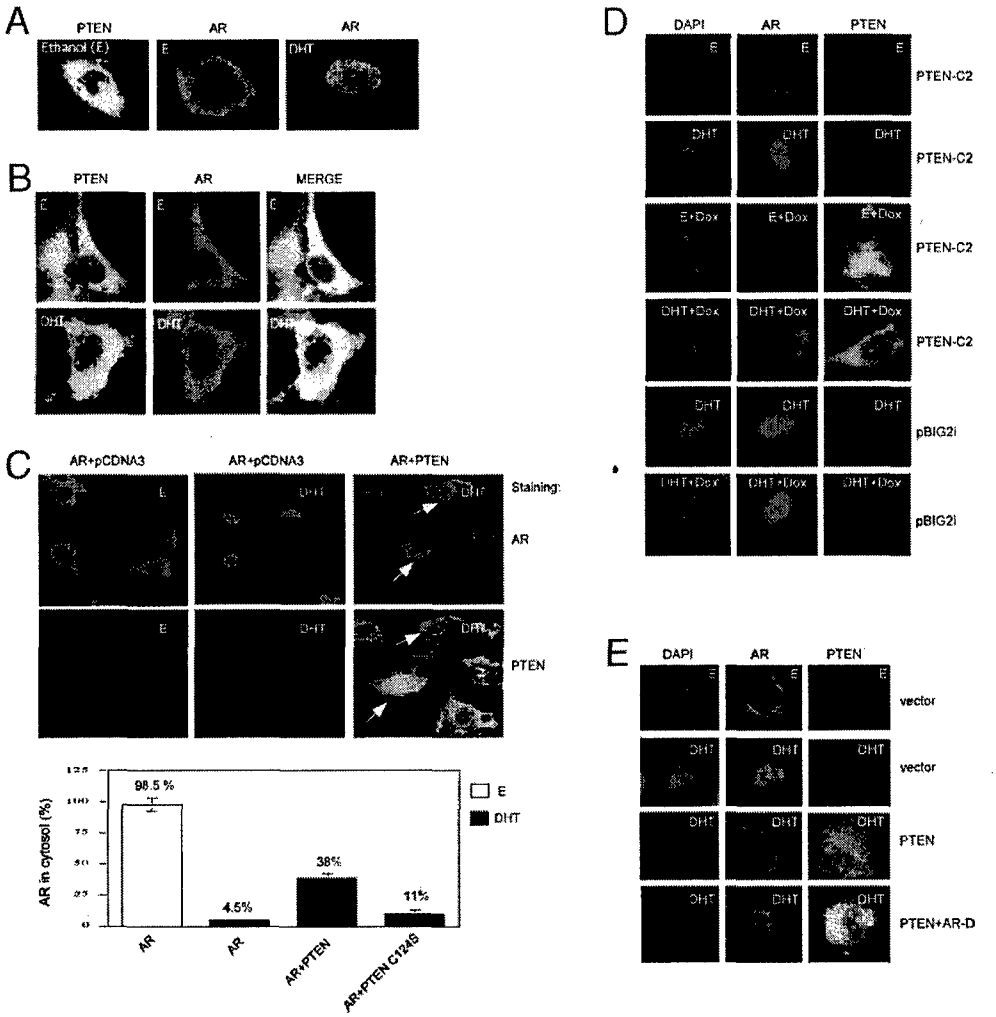

Fig. 4. PTEN Colocalizes with AR *in Vivo* and Prevents AR Nuclear Translocation

A, The COS-1 cells were transfected with AR or PTEN in 10% CDS media for 16 h and treated with ethanol or 10 nM DHT for another 16 h. The cells were fixed and stained with AR and PTEN antibodies, followed by examination with confocal microscopy. B, The COS-1 cells were transfected with AR and PTEN and treated with ethanol or 10 nM DHT for another 16 h. The cells were fixed and stained with AR and PTEN antibodies, followed by examination with confocal microscopy. The *green and red* colors represent PTEN and AR staining, respectively, and the *yellow* color represents PTEN and AR colocalization. C, COS-1 cells were transfected with pSG5-AR along with pCDNA3, pCDNA3 PTEN, or PTEN C124S and treated with ethanol or DHT in 10% CDS media for 16 h. The *arrows* indicate PTEN-positive cells, which show AR located in the cytosol. At least 150 cells were scored for each sample, and data are means ± SD from three independent experiments. D, The pBIG2i or PTEN-C2 LNCaP cells were treated with 4 μg/ml Dox for 24 h, followed by 10 nM DHT for another 16 h. The cells were fixed for immunostaining. E, The COS-1 cells were transfected with AR in combination with plasmids, as indicated on the *right* for 16 h, followed by 10 nM DHT treatment for another 16 h. The cells were fixed for immunostaining.

PTEN Decreases AR Protein Levels via Promotion of AR Degradation

To determine whether PTEN suppression of AR transactivation involves the modulation of AR protein stability, we assessed the transient transfection and Western blot analyses. We found that PTEN could reduce AR protein levels in COS-1 cells (Fig. 5A). To rule out the possibility that PTEN may influence the promoter activity of the AR expression plasmid, we tested the expression of endogenous AR in PTEN-stable LNCaP cells. As shown in Fig. 5B, Dox-induced expression of PTEN in LNCaP PTEN-C1 and PTEN-C2 reduced endogenous AR protein levels. In contrast, Dox-induced PTEN C124S expression in PTEN-C124S-C4 and PTEN-C124S-C8 failed to reduce endogenous AR protein levels. Together, our data clearly demonstrate that PTEN could interact with AR and reduce AR protein levels in COS-1 and LNCaP cells. To determine whether the reduced AR protein levels were due to reduced mRNA expression, a portion of each LNCaP cell lysate was subjected to Northern blot analysis. Whereas AR protein levels were reduced by Dox-induced PTEN, the AR mRNA

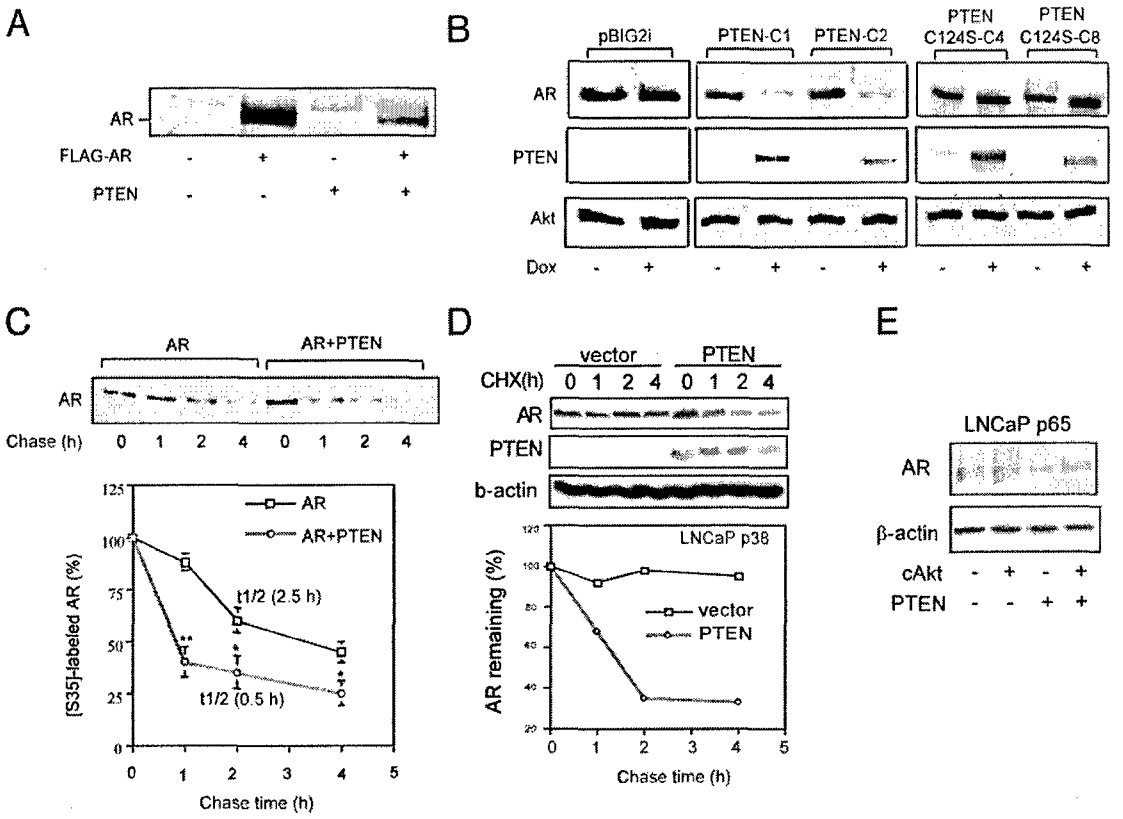

Fig. 5. PTEN Decreases AR Protein Levels via Promotion of AR Degradation

A, COS-1 cells were transfected with AR with a flag epitope in front of the AR sequence, along with pCDNA3 or PTEN in 10% CDS media for 16 h, followed by treatment with 10 nM DHT for 24 h. The cells were harvested for Western blot analysis with anti-FLAG antibody. B, Clones of LNCaP cells stably transfected with vector (pBIG2i), PTEN (PTEN-C1 and -C2), or PTEN-C124S (PTEN C124S-C4 and -C8) were treated with 4 μg/ml doxycycline in 10% CDS media for 48 h in the presence of 10 nM DHT. Western blot analysis was performed, and AR and PTEN were detected by AR antibody or PTEN antibody, respectively. C, COS-1 cells were transfected with AR along with pCDNA3 or PTEN in 10% CDS media for 16 h. The cells were then pulsed with [^{35}S]methionine for 45 min in the presence of 10 nM DHT and harvested at different chase times as indicated. The cell extracts were immunoprecipitated with AR antibody and subjected to SDS-PAGE followed by autoradiography. The intensity of the bands was quantitated using ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA). Data were from three identical results. D, Early-passage LNCaP cells at passage 38 (p38) were transfected with or without PTEN in 10% CDS media for 16 h, pulsed with cyclohexamide (CHX) treatment in the presence of 10 nM DHT, and then harvested at different chase times as indicated. The cell extracts were Western blotted with AR, PTEN, and β-actin antibodies. E, PTEN-regulated AR degradation is inhibited by Akt in high-passage number LNCaP cells. LNCaP cells at passage 65 (p65) were transfected with plasmids, as indicated, for 24 h, treated with 10 nM DHT for another 24 h, and harvested for Western blot assay. (*, $P < 0.05$; **, $P < 0.001$ *vs.* AR alone, Student's two-tailed *t* test)

levels normalized by β-actin remained relatively unchanged (data not shown), suggesting that PTEN may reduce the AR protein levels through posttranscriptional modification.

We then studied AR protein stability by pulse chase labeling. As shown in Fig. 5C, PTEN clearly reduced the half-life of newly synthesized [^{35}S]AR 4- to 5-fold and accelerated AR degradation. Interestingly, when we replaced PTEN with either the dominant negative form of Akt (dAkt) or PI3K inhibitor LY294002, the results (data not shown) indicated that dAkt and LY294002 did not promote AR degradation, ruling out the possibility that PTEN promotes AR degradation via regulation of the PI3K/Akt pathway. In agreement with the phenomenon that PTEN promotes AR degradation via the non-PI3K/Akt pathway, the stability of the endogenous AR in the early-passage LNCaP cells (passage 38), where the PTEN effect on AR is suggested to be independent of the PI3K/Akt pathway (Fig. 1E), was clearly reduced in the presence of PTEN (Fig. 5D). These data strongly suggest that other pathways, such as direct PTEN-AR protein-protein interaction, may play major roles for the PTEN-promoted AR degradation. In contrast, in high passage number LNCaP cells (passage 65) where the PI3K/Akt pathway becomes dominant (Fig. 1, D and E), PTEN-induced AR degradation was suppressed by cAkt (Fig. 5E), suggesting that the suppressive effect of PTEN on AR

involves the Akt pathway and Akt might not promote AR ubiquitylation and degradation in high-passage LNCaP cells.

It has been suggested that PTEN regulates the stability of $p27^{kip1}$ via a ubiquitin-proteasome pathway (38). Whereas MG132, a proteasome inhibitor, blocked estrogen-mediated ER degradation (see supplemental Fig. 1A, *right panel*, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org), it did not prevent PTEN-mediated AR degradation (see supplemental Fig. 1A, *left panel*), suggesting that PTEN promotes AR protein degradation via a proteasome-independent pathway. Interestingly, we found that the caspase-3 inhibitor DEVD-CHO, can block PTEN-mediated AR degradation (see supplemental Fig. 1B). We demonstrated that caspase-3 could cleave AR into three evident fragments, and DEVD-CHO completely blocked caspase-3-mediated AR degradation (see supplemental Fig. 1B), consistent with the previous reports (39).

Interaction between PTEN and AR Contributes to PTEN-Induced Suppression of AR Functions and Apoptosis

To further prove that PTEN suppression of AR function may go through direct PTEN-AR interaction, we used AR-D, which can interact with PTEN and disrupt the interaction between AR and PTEN in the CWR22R cells (Fig. 3D), for functional studies. Our results further showed that AR-D could dramatically reduce PTEN-mediated inhibition of AR nuclear translocation (Fig. 4E), PTEN-mediated promotion of AR degradation (Fig. 6A), and PTEN-mediated suppression of AR transactivation (Fig. 6B), suggesting that PTEN and AR interaction plays important roles for the PTEN effects on AR nuclear translocation, AR protein degradation, and AR transactivation. To extend our studies of PTEN on the suppression of AR function, we applied AR-D to another prostate cancer cell line, CWR22R, which expresses functional AR and PTEN (36, 37). As shown in Fig. 6C, PTEN dramatically suppressed AR transactivation. Remarkably, AR-D could significantly reduce PTEN suppressive effect on AR transactivation (Fig. 6C). Furthermore, the PTEN mutant devoid of AR binding region (Δ aa121–200, PTEN-dPTP) failed to suppress AR expression (Fig. 6D) and transactivation (Fig. 6E). These results therefore are in agreement with the results from LNCaP cells (Fig. 6B) and suggest that PTEN may be able to modulate AR functions by direct interaction with AR in the various stages of prostate cancers.

The PTEN tumor suppressor induces cell apoptosis in a variety of cell types including the LNCaP cells. To determine whether suppression of AR activity by PTEN contributes to PTEN-induced apoptosis, we used the TUNEL (terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labeling) assay to study the effect of AR-D, which could partially relieve suppression of AR transactivation by PTEN (Fig. 6, B and C), on PTEN-induced apoptosis in LNCaP cells (passage 35). As expected, PTEN could induce apoptosis markedly, whereas PTEN C124S showed only a marginal effect, and AR-D markedly reduced PTEN-induced apoptosis (Fig. 6F). The suppressive effect of AR-D on PTEN functions was not due to the interference of PTEN phosphatase activity, because AR-D showed little influence on the PTEN-mediated inhibition of Akt activity (data not shown). Together, these data clearly suggest that interaction between PTEN and AR contributes to PTEN-induced suppression of AR functions and apoptosis. Our results (Fig. 6F) also confirmed an earlier report (39a) that PTEN-induced cell death could be reversed by adding cAkt, suggesting that the PTEN→ PI3K→ Akt pathway also plays a role in the mediation of PTEN-induced cell death.

To rule out the possibility that AR-D may have nonspecific effects, we used glioblastoma U87MG cells to test the effects of AR-D on the PTEN-induced apoptosis in AR-negative cells. Both Western blot assay and AR transactivation assay indicated that AR was undetectable in U87MG cells (data not shown). Whereas PTEN induced apoptosis in U87MG cells, the addition of AR-D showed only marginal effects on the PTEN-induced apoptosis, and cAkt suppressed PTEN-induced apoptosis (data not shown). These results suggest that the effect of AR-D on PTEN-induced apoptosis is specific and requires the intact AR signaling. Together, results from Fig. 6 clearly indicate that PTEN may have two distinct pathways (PTEN→PI3K/Akt and PTEN→AR) to induce apoptosis, and the interaction of PTEN with AR may play important roles in one of these two pathways in the LNCaP prostate cancer cells.

Inhibition of Endogenous PTEN Expression Increases AR Protein Levels and Transcriptional Activity

We have demonstrated that overexpression of PTEN promotes AR degradation and suppresses AR activity. To avoid the above observations resulting from overexpression, we used small interfering RNA (siRNA) to block endogenous PTEN and examined whether the AR protein levels and transcriptional activity would be affected by down-regulating PTEN. As shown in Fig. 7A, transient transfection of PTEN siRNA into human embryonic kidney 293T cells reduced endogenous PTEN protein levels up to 50–60%, which correlated with the transfection efficiency (~50%) in our experiment conditions. As expected, reduction of PTEN expression enhanced AR protein expression in the presence and absence of androgen (Fig. 7A). PTEN siRNA enhanced AR transactional activity in a dose-dependent manner in the presence of androgen in 293T cells (Fig. 7B). Furthermore, we observed that the levels of AR protein expression in PTEN-null MEFs were much higher than that in WT MEFs (Fig. 7C), suggesting that AR may be more stable in the absence of PTEN. Reintroduction of PTEN in PTEN-null

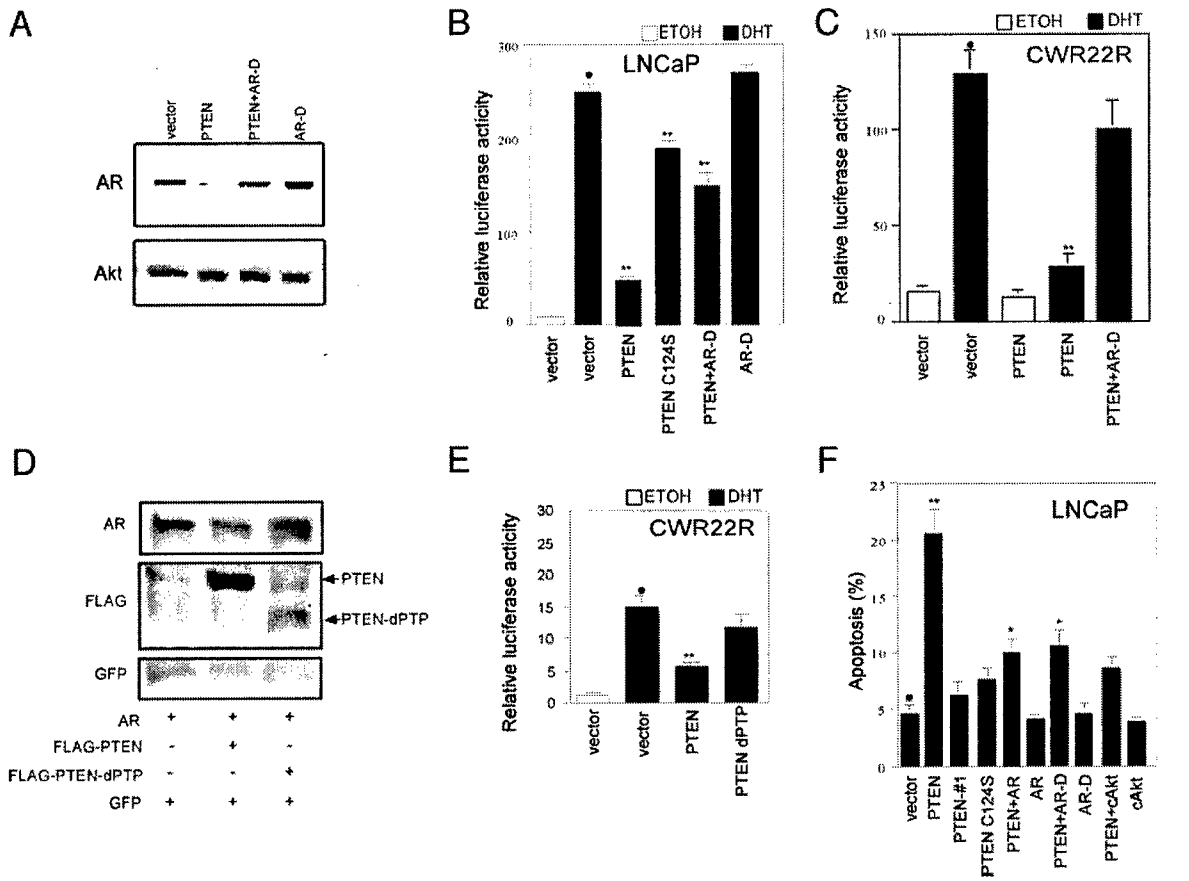

Fig. 6. Interaction between PTEN and AR Contributes to PTEN-Mediated Apoptosis and Suppression of AR Functions

A, The LNCaP cells were transfected with indicated plasmids in 10% CDS media for 16 h, followed by treatment with 10 nM DHT for 24 h. Cells were then harvested, and the cell extracts were subjected to Western blotting with anti-AR or anti-Akt antibody. B, The LNCaP cells were transfected with indicated plasmids in 10% CDS media for 16 h, followed by treatment with 10 nM DHT for another 16 h. Cells were harvested and assayed for MMTV-luciferase activity. C, The CWR22R cells were transfected with plasmids, as indicated, using (ARE)4-luc as a reporter for 16 h, followed by ethanol or 10 nM DHT treatment for another 16 h. Cells were harvested for luciferase assay. D, The 293T cells cultured in DMEM containing 10% FCS were transfected with pCDNA3-AR, pCDNA3-FLAG-PTEN, pCDNA3-FLAG-PTEN-dPTP, and/or pEGFP-C1 (BD Biosciences, Franklin Lakes, NJ), as indicated, for 24 h, and harvested. The cell extracts were subjected to SDS-PAGE. Western blot analysis was performed, and AR and PTENs were detected by AR and FLAG antibodies, respectively. Enhanced green fluorescent protein expression was used for transfection and loading control. E, The CWR22R cells were transfected with plasmids, as indicated, using (ARE)4-luc as a reporter for 16 h, followed by ethanol or 10 nM DHT treatment for another 16 h. Cells were harvested for luciferase assay. F, The LNCaP cells were transfected with plasmids, as indicated, for 16 h, and the medium was changed to 0.1% CDS media for 2 d. The cell apoptosis was determined by TUNEL assay. PTEN, but not PTEN-no. 1 (aa 1~107) or mutant PTEN-C124S, induced LNCaP cell apoptosis. Increased AR expression by transfection of AR, interrupting PTEN-AR interaction by AR-D, and overexpressing cAkt could rescue LNCaP cell apoptosis caused by PTEN. Data for luciferase activity and apoptosis are means ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.001$ *vs.* control (indicated as ●), Student's two-tailed *t* test.

MEFs drastically reduced AR protein levels, as compared with that in WT MEFs (Fig. 7C). We also found that AR transcriptional activity in PTEN-null MEFs was much higher than in WT MEFs, and reconstitution of PTEN in PTEN-null MEFs significantly suppressed AR activity (Fig. 7D). cAKt did not reverse the PTEN-mediated repression of AR activity (Fig. 7D), suggesting that PTEN suppresses AR activity via the PI3K/Akt-independent pathway in MEFs. These results suggest that endogenous PTEN is a negative regulator for controlling AR protein stability and transcriptional activity.

DISCUSSION

The PTEN tumor suppressor induces cell apoptosis in a variety of cell types including the prostate cancer cells. However, the molecular mechanism underlying PTEN-induced apoptosis in prostate cancers remains largely unknown. In the present study we have identified AR as a novel target of PTEN *in vitro* and *in vivo*. PTEN inhibited AR nuclear translocation, promoted AR protein degradation, and inhibited AR transactivation via direct PTEN-AR interaction. We also demonstrated

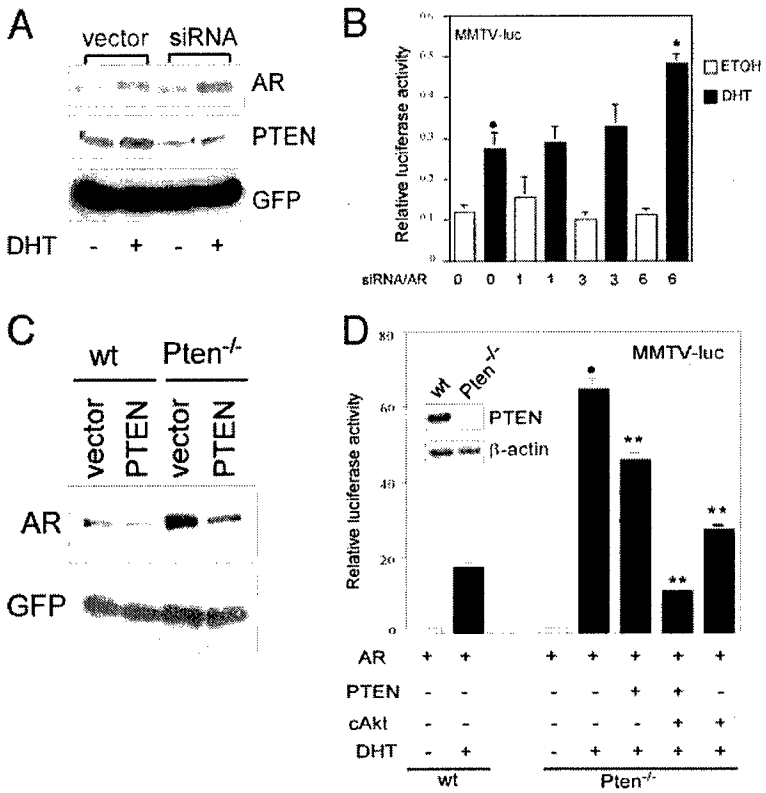

Fig. 7. Endogenous PTEN Negatively Regulates AR Protein Stability and Transcriptional Activity

A, 293T cells were transfected with PTEN siRNA or vector along with AR and green fluorescent protein (GFP) for 24 h, followed by ethanol or 10 nM DHT treatment for another 24 h, and harvested for Western blot analysis. GFP expression was used for transfection and loading control. B, 293T cells were transfected with various amounts of PTEN siRNA or vector along with AR and MMTV-luc for 24 h, followed by ethanol or 10 nM DHT treatment for another 24 h, and harvested for luciferase assay. C, WT and PTEN-null MEFs were transfected with AR and GFP in the presence or absence of PTEN for 36 h and harvested for Western blot analysis. GFP expression was used for transfection and loading control. D, WT and PTEN-null MEFs were transfected with plasmids as indicated for 24 h, followed by ethanol or 10 nM DHT treatment for another 24 h, and harvested for luciferase assay. *, $P < 0.05$; **, $P < 0.001$ *vs.* control (indicated as ●), Student's two-tailed *t* test.

that PTEN-induced suppression of AR transactivation and apoptosis could be inhibited by interruption of PTEN and AR interaction by adding AR-D peptide. Furthermore, using the PTEN siRNA and PTEN-null MEFs we found that AR protein levels and transcriptional activity were elevated. Taken together, these results indicate that PTEN is a negative regulator for controlling the AR activity and that interaction between AR and PTEN may play an important role for PTEN to suppress AR and induce apoptosis in prostate cancer cells.

Like other members of the steroid receptor superfamily, AR may move dynamically between the nucleus and the cytoplasm (40). As androgen induces AR nuclear translocation and prolongs the half-life of AR (41), it is generally accepted that AR degradation might be prevented by binding androgen and translocating into the nucleus. Because PTEN suppresses AR nuclear translocation and promotes AR degradation, it is possible that these two events occur subsequently and are functionally linked. It is likely that PTEN may first bind to AR, leading to retention of this PTEN-AR complex in the cytoplasm, which may then make AR more vulnerable to enzymatic degradation.

Sequence analysis indicates that AR contains the nuclear localization signal (NLS) (aa 617 to 633) in the hinge region between DBD and LBD. It has been reported that mutations in the NLS may lead to the disruption of AR nuclear translocation (42). It is plausible that PTEN binds to the hinge region of AR resulting in the interruption of the NLS nuclear translocation. Alternatively, PTEN could simply compete with other AR coregulators for binding to the same region, causing the inhibition of the nuclear translocation.

Recent reports suggest that PTEN may exert its biological activity by regulating stability of proteins. For example, PTEN can regulate the ubiquitin-dependent degradation of CDK inhibitor $p27^{Kip1}$ through the ubiquitin E3 ligase SCF^{Skp2} (38). PTEN can also attenuate the hypoxia-mediated HIF-1α (hypoxia-inducible factor 1) stabilization (43). Together with our finding showing that PTEN promotes AR degradation, these results support a role of PTEN in modulation of protein degradation. Proteins containing the PEST sequence

are thought to be the target proteins for ubiquitination and degradation (44). As AR contains the PEST sequence (aa 638 to 658) in the hinge region (45), it is possible that PTEN accelerates AR degradation through the ubiquitin-proteosome pathway via the PEST sequence. However, this hypothesis is not supported by our result showing that PTEN-induced AR degradation is not affected by the treatment of the proteasome inhibitor MG132 (see supplemental Fig. 1A), suggesting the unlikelihood that PTEN goes through the ubiquitin-proteasome pathway to promote AR degradation. As the caspase-3 inhibitor completely blocked the effect of PTEN on AR degradation (see supplemental Fig. 1A), caspase-3 may mediate the PTEN-induced AR degradation. This hypothesis is further supported by our result (see supplemental Fig. 1B) and the earlier report showing that caspase-3 could degrade AR *in vitro* (39). It has been reported that PTEN-induced apoptosis can be rescued by caspase-3 inhibitor in LNCaP cells (46), which also strengthens our hypothesis that PTEN signaling can be mediated through caspase-3 via direct cleavage of AR protein (39). Although our study demonstrates that PTEN-mediated AR degradation is through caspase-3 activity (see supplemental Fig. 1), we found that the repression of AR activity by PTEN could not be rescued by a caspase-3 inhibitor or a general caspase inhibitor (see supplemental Fig. 1C). These contrasting results imply that PTEN suppression of AR might go through multiple pathways, and caspase-3-mediated degradation could be one of these pathways. In addition, because it has been known that caspase-3 cleaves AR at the D151 residue, we further tested the effect of PTEN on AR-D151N mutant. We found that PTEN can still repress the transactivation of AR-D151N (see supplemental Fig. 1D), indicating that PTEN suppressed AR not only via protein degradation. Because PTEN-dPTP (lacking AR interacting domain) failed to suppress AR transactivation (Fig. 6E) and AR-D (interaction inhibitor) can block the suppressive effect of PTEN on AR transactivation (Fig. 6, A–C and F), it is possible that in addition to degradation of AR, direct association between AR and PTEN may also contribute to suppression of AR activity.

We reported recently that the PI3K/Akt pathway promoted AR ubiquitylation, leading to AR degradation by the 26 S proteasome (47). These data clearly suggest that both PTEN and the PI3K/Akt pathway can promote AR degradation via distinct mechanisms. How can PTEN negatively regulate the PI3K/Akt pathway and at same time promote AR degradation? Because PI3K/Akt signaling promotes AR degradation, PTEN inhibition of this pathway would be expected to result in increased AR protein levels. It is possible that PTEN can go through both pathways by inhibition of PI3K/Akt-mediated AR degradation by the 26 S proteasome and caspase-3-mediated AR degradation. Yet the overall balance may favor the caspase-3-mediated AR degradation.

Because the interaction between PTEN and AR plays an important role in PTEN-mediated AR degradation (Fig. 5A), it is possible that PTEN binding to AR may be required to expose the active site of the AR for caspase-3 recognition, thus leading to AR degradation. This hypothesis is supported by the demonstration that some apoptosis inducers, such as staurosporine and phorboester (phorbol myristate acetate), can induce caspase-3 activation (48, 49), but fail to induce AR degradation (data not shown) (50).

A mutant PTEN C124S, which does not have phosphatase activity, exhibits a significantly reduced ability to suppress AR activity in LNCaP and DU145 cells, indicating that PTEN phosphatase activity is important for PTEN-mediated AR suppression. Given that PTEN directly interacts with AR and that its phosphatase activity is important for its effect on AR activity, it is possible that PTEN may regulate AR activity via direct dephosphorylation of AR. However, we were unable to detect a significant change in AR phosphorylation upon addition of GST-PTEN in an *in vitro* dephosphorylation assay (data not shown). These data raise the possibility that PTEN may regulate AR activity, in part, via indirectly affecting the phosphorylation status of other proteins.

The loss of PTEN expression in prostate LNCaP cells leads to constitutive activation of Akt (15). Akt is an important survival factor in a variety of cell types including LNCaP cells (15). Several lines of evidence have indicated that PI3K/Akt is able to suppress cell apoptosis induced by growth factor deprivation (16, 51, 52). Abrogation of PI3K/Akt activity by PI3K inhibitors causes LNCaP cell apoptosis (53, 54). On the other hand, the androgen/AR signal is thought to play important roles in the prostate cancer cell growth and survival, and this signal can protect cells from apoptosis in response to treatment with PI3K inhibitors (54, 55). Thus, the PI3K/Akt and the androgen/AR signaling pathways represent two major survival pathways in the LNCaP prostate cancer cells. As PTEN could repress the androgen/AR signal and PI3K/Akt pathway in LNCaP cells, we propose that inhibition of these two pathways by PTEN might contribute to PTEN-induced cell apoptosis in the LNCaP prostate cancer cells. This assertion was further supported by the observation that restoration of AR function or the PI3K/Akt pathway rescues cells from PTEN-induced apoptosis (Fig. 6F).

Consistent with the reporter gene assay (Fig. 1E, *right panel*), in the high-passage number LNCaP cells we found that PTEN could down-regulate AR protein levels and this effect was reversed by Akt (Fig. 5E). Furthermore, Akt did not down-regulate AR protein levels (Fig. 5E), suggesting that Akt might not promote AR ubiquitylation and degradation in high-passage LNCaP cells. Based on our data we propose a model for PTEN action on AR signaling in prostate cancer LNCaP cells. PTEN regulates AR activity in low-passage LNCaP cells via a PI3K/Akt-independent pathway and interacts directly with AR to suppress

androgen-induced AR nuclear translocation. The interaction between AR and PTEN might expose the active site of the AR for the recognition of caspase-3. The PTEN activated caspase-3 then recognizes the AR and leads to AR degradation (Fig. 8). Although overexpression of the active form of Akt can inhibit PTEN-induced caspase-3 activation, thus potentially blocking PTEN-mediated AR degradation, Akt itself can induce AR degradation. This may explain why restoration of Akt activity does not reverse PTEN-mediated AR suppression and AR degradation in the early-passage number LNCaP cells. In contrast, PTEN promotes AR degradation and suppress AR activity in high-passage number LNCaP cells via a PI3K/Akt-dependent pathway (Fig. 8). In such cells, Akt does not down-regulate AR protein levels (perhaps not inducing AR degradation), and it may account for the reason why PTEN-induced suppression of AR activity and AR degradation are inhibited by restoration of Akt activity. Several important questions have been raised throughout this study. First, what is the factor(s) that determines the differential effects of the PI3K/Akt pathway on AR activity in different passage numbers of LNCaP cells? What factor(s) triggers the distinct mechanisms used by PTEN to regulate AR activity in various passage numbers of LNCaP cells? What factor(s) can be dephosphorylated by PTEN and also contribute to PTEN-mediated AR suppression? Future studies should focus on these issues, and systematic analysis is required to solve these puzzles.

MATERIALS AND METHODS

Constructs and Reagents

pCDNA3-cAkt and pCDNA3-dAkt were from Dr. R. Freeman. LY294002, DEVD-CHO, and z-VAD-FMK were from Calbiochem (La Jolla, CA). 5α-Dihydrotestosterone (DHT) and Dox were from Sigma Chemical Co. (St. Louis, MO). The anti-AR polyclonal antibody, NH27, was produced as previously described (8, 56). Recombinant active caspase-3 was purchased from Pharmingen (San Diego, CA). PTEN monoclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). FITC-conjugated secondary antimouse antibody and Texas Red-conjugated secondary antirabbit antibody were from ICN Biochemicals, Inc. (Aurora, OH). TNT *in vitro* protein expression kit was from Promega. pCDNA3-PTEN and pCDNA3-PTEN C124S were provided by Dr. C. L. Sawyers. pSG5-HA-PTEN was from Dr. W. Sellers. PTEN-no. 1 (encoding PTEN aa 1–107) and AR-D (encoding AR aa 486–651) were constructed into pCMV-FLAG vector. To construct GST-PTEN fragment proteins, the PTEN fragments (nos. 1, 2, and 3) were released from pGEX-KG-PTEN (from Dr. F. Furnari) and subcloned into pGEX-KG, pGEX-2t, and pGEX-3x (Amersham Pharmacia Biotech, Arlington Heights, IL), respectively. To construct GST-PTEN-PTP, the PTP fragment was obtained by PCR and inserted into pGEX-3x. pCDNA3-FLAG-PTEN and pCDNA3-FLAG-PTEN-dPTP (lacking PTEN aa 110–200) were generated by PCR, and the cDNAs were inserted into pCDNA3 vector (Invitrogen).

Cell Culture and Transfections

The DU145, PC-3, 293T, COS-1, and the wild-type (WT) and PTEN-null MEFs (kindly provided by Dr. H. Wu) were maintained in DMEM containing penicillin (25 U/ml), streptomycin

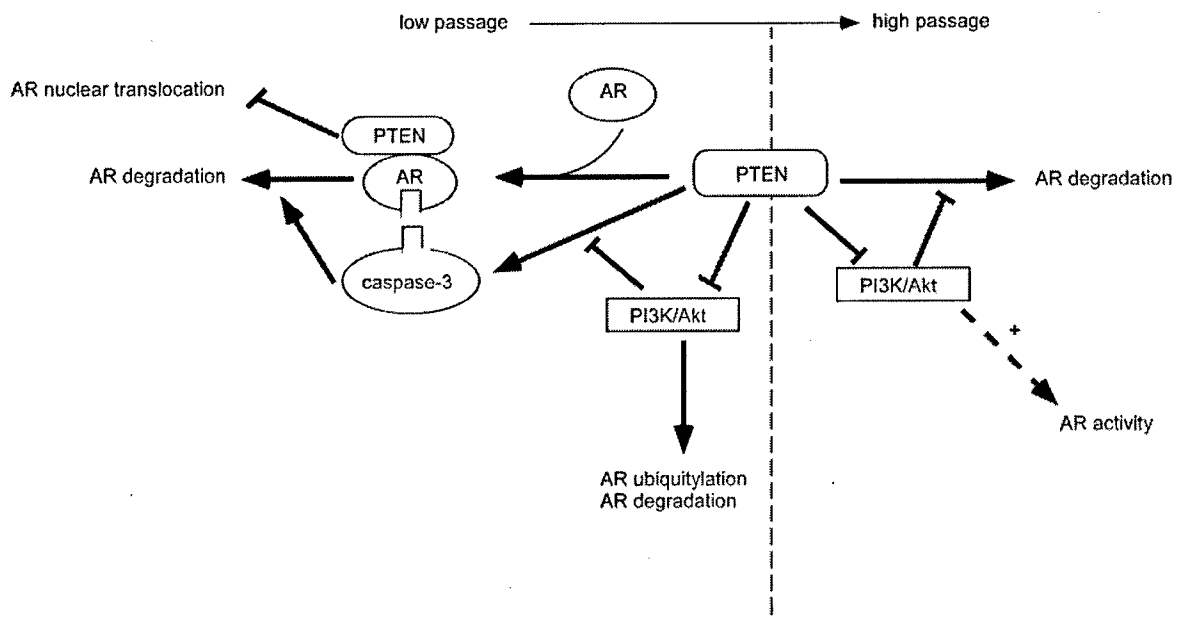

Fig. 8. A Model for the PI3K/Akt Pathway and PTEN Tumor Suppressor on AR Signaling in Prostate LNCaP Cells

In low-passage number LNCaP cells, the PI3K/Akt pathway suppresses AR activity and induces AR ubiquitylation and degradation by 26S proteasome (47). PTEN tumor suppressor also suppresses AR activity at this early-passage number of LNCaP cells via a PI3K/Akt-independent pathway. PTEN directly interacts with AR and induces caspase-3 activation. The interaction between AR and PTEN may lead AR to expose the active site for caspase-3 recognition, resulting in AR degradation. Furthermore, the interaction between AR and PTEN results in suppression of AR nuclear translocation. However, the PI3K/Akt enhances AR activity in high-passage number LNCaP cells via an unknown mechanism. PTEN, on the other hand, suppresses AR activity via a PI3K/Akt-dependent pathway.

(25 μg/ml), and 10% fetal calf serum (FCS). The LNCaP, U87MG, and CWR22R (a gift from Dr. C. Kao) cells were maintained in RPMI-1640 with 10% FCS. Transfections were performed using the calcium phosphate precipitation method in PC-3 and DU145 cells, as previously described (57) or SuperFect in LNCaP, COS-1, and U87MG cells according to standard procedures (QIAGEN, Chatsworth, CA).

Apoptosis Assay

For the apoptosis assay, the cells were transfected with plasmids for 24 h and grown in 0.1% charcoal dextran-stripped serum (CDS) media. The apoptosis was determined 2 d after transfection using the TUNEL assay according to the standard procedures (Oncogene Science, Inc., Manhasset, NY). At least 200 cells were scored for each sample, and data are means ± SD from three independent experiments.

Luciferase Reporter Assays

The cells were transfected with plasmids in 10% CDS media for 16 h and then treated with ethanol or 10 nM DHT for 16 h. The cells were lysed and the luciferase activity was detected by the dual luciferase assay according to standard procedures (Promega). The results were normalized by pRL-SV40 activity, and the data are represented as means ± SD from at least three independent experiments.

Glutathionine-S-transferase (GST) Pull-Down Assay

The GST pull-down assay described previously (58) was performed with some modifications. GST-fusion proteins were purified as described by the manufacturer (Amersham Pharmacia Biotech). The purified GST-proteins were resuspended with 100 μl of interaction buffer [20 mM Tris-HCl (pH 8.0), 60 mM NaCl, 1 mM EDTA, 6 mM $MgCl_2$, 1 mM dithiothreitol, 8% glycerol, 0.05% (vol/vol) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors] and mixed with 5 μl of ^{35}S-labeled TNT proteins in the presence or absence of 1 μM ligands on a rotating disk at 4 C for 2 h. After extensive washes with NENT buffer (20 nM Tris-HCl/pH 8.0, 100 mM NaCl, 6 mM $MgCl_2$, 1 mM EDTA, 0.5% (vol/vol) Nonidet P-40, 1 mM dithiothreitol, 8% glycerol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors) bead-bound proteins were separated on an 8% sodium dodecyl sulfate-polyacrylamide gel and visualized by autoradiography.

Immunoprecipitation and Western Blot Analysis

The immunoprecipitation and Western blotting were performed as previously described (59). The cell extracts (1 mg) were immunoprecipitated with the indicated antibody. The immunocomplexes were subjected to 8% SDS-PAGE and immunoblotted with the indicated antibodies.

Immunofluorescence and Microscopy

The COS-1 cells were plated on 12-mm coverslips, incubated overnight, and transfected with pSG5-AR in combination with pCDNA3, pCDNA3 PTEN, or pCDNA3 PTEN C124S in 10% CDS media for 16 h, followed by the treatment with ethanol or 10 nM DHT for another 16 h. The cells were fixed with 4% paraformaldehyde/PBS for 20 min on ice and permeabilized with 100% methanol for 15 min on ice. The following experiments were performed at room temperature. The coverslips were rinsed with PBS twice and incubated in 5% BSA for 30 min. The primary antibodies against AR and PTEN were added for 1 h and then washed with PBS four times. The secondary antibodies were added for 1 h, and the coverslips were washed four times with PBS, followed by application of the counting medium containing 4′,6-diaminodino-2-phenylindole. Coverslips were examined by confocal microscope. A FITC-conjugating antimouse antibody and a Texas Red antirabbit antibody were used as secondary antibodies.

LNCaP Stable Transfectants

For the Dox-inducible system, PTEN or PTEN C124S were released from pGEX-KG-PTEN or pGEX-KG-PTEN C124S using Eco*RI* digestion and inserted into pBIG2i vector. The LNCaP cells were transfected with pBIG2i vector, pPIB2i PTEN, or pBIG2i PTEN C124S for 24 h. The cells were selected with 100 μg/ml hygromycin B. Individual colonies were picked up and grown until 70% confluent, followed by 4 μg/ml Dox treatment for 48 h. The positive clones were confirmed by Western blot analysis.

Pulse-Chase Experiments

Pulse-chase experiments were performed as described (60) with some modifications. Briefly, COS-1 cells were transfected with pSG5-AR in combination with pCDNA3 or pCDNA3 PTEN in 10% CDS media for 36 h. Cells were grown under serum starvation conditions for 2 h in methionine/cysteine-deficient medium, and then the cells were pulsed for 45 min with 200 μCi/ml [^{35}S]methionine/cysteine (NEN Life Science Products, Boston, MA). Cells were washed with DMEM twice and incubated with DMEM containing 0.2% CDS along with 10 nM DHT. The cells were lysed by RIPA buffer in the presence of protease inhibitors, followed by immunoprecipitation using AR antibody. The immunocomplexes were subjected to 8% SDS-PAGE and visualized by autoradiography.

Construction of PTEN siRNA

A small-interfering RNA (siRNA) was expressed in mammalian cells by transfection of a DNA-based vector BS/U6 (61) containing a homologous sequence (CCCACCACAGCTAGAACTTATC), a 6-bp spacer (CTCGAG), an inverted homologous sequence (GATAAGTTCTAGCTGTGGTGGG), and 5 "T"s, at the transcription initiation site of the U6 promoter.

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Address all correspondence and requests for reprints to: Chawnshang Chang, Ph.D., Department of Pathology, University of Rochester Medical Center, 601 Elmwood Avenue, Box 626, Rochester, New York 14642. E-mail: chang@urmc.rochester.edu.

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